NATAN DE JESUS PIMENTEL FILHO

EFFECT OF BACTERIOCINS ON THE ADHESION AND PROTEIN EXPRESSION PROFILING OF *Staphylococcus aureus*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 25 de fevereiro de 2014.

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(Coorientador)

Denise Mara Soares Bazzolli

Maria Cristina Baracat-Pereira

Stephan Fuchs

Maria Cristina Dantas Vanetti
(Orientadora)

Às mulheres da minha vida,
Minha mãe Teresinha
Minha irmã Caroline
Minha noiva Larissa

DEDICO.

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BIOGRAFIA

NATAN DE JESUS PIMENTEL FILHO, filho de Natan de Jesus Pimentel e Teresinha Rizzo Pimentel, nasceu em Itabira, Minas Gerais no dia 06 de janeiro de 1983.

Em maio de 2002 iniciou o curso de Ciência e Tecnologia de Laticínios na Universidade Federal de Viçosa, tornando-se bacharel em outubro de 2006.

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Em março de 2010, iniciou o curso de Doutorado em Microbiologia Agrícola na Universidade Federal de Viçosa, concentrando seus estudos na mesma área. Participou do Programa Doutorado Sanduíche no Exterior/Capes na Ernst-Moritz-Arndt-Universität Greifswald, Alemanha, de abril de 2012 a março de 2013. Submeteu-se à defesa de tese em 25 de fevereiro de 2014.

SUMÁRIO

RESUMO

PIMENTEL FILHO, Natan de Jesus, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Efeito de bacteriocinas sobre a adesão e o perfil de expressão de proteínas de *Staphylococcus aureus***. Orientador: Maria Cristina Dantas Vanetti. Coorientador: Hilário Cuquetto Mantovani.

Staphylococcus aureus é um patógeno humano oportunista responsável por uma série de doenças que vão desde intoxicações alimentares e infecções superficiais da pele a doenças mais graves como endocardite, pneumonia, meningite, sépsis e a síndrome do choque tóxico. *S. aureus* é capaz de aderir a superfícies e formar biofilmes e, na indústria de alimentos, esses biofilmes podem levar à contaminação cruzada causando riscos à segurança alimentar. O objetivo deste estudo foi avaliar o efeito de concentrações subinibitórias das bacteriocinas bovicina HC5 e nisina sobre a adesão de *S. aureus* em superfície de poliestireno e, por meio da proteômica, avaliar a resposta ao estresse deste patógeno à bovicina HC5. Doses subinibitórias das bacteriocinas, que não impediram o crescimento bacteriano, reduziram a adesão de células, provavelmente por alterar a hidrofobicidade das superfícies da bactéria e do poliestireno. Ambas as superfícies tornaram-se mais hidrofílicas e a energia livre foi termodinamicamente desfavorável à adesão após o tratamento com bovicina HC5 e nisina. O nível de transcritos de genes relacionados com adesão e mecanismo de *quorum sensing* foi avaliado por RT-qPCR e revelou que as bacteriocinas afetaram a expressão dos genes *icaD, fnbA* e *clfB*, relacionados à formação de biofilmes e também, do gene *rnaIII*, envolvido no mecanismo de *quorum sensing*. A expressão de *icaD* e *fnbA* foi aumentada na presença das bacteriocinas avaliadas. Enquanto bovicina HC5 diminuiu a expressão de *clfb*, o nível de transcritos de *rnaIII* aumentou na presença de nisina. O perfil de síntese de proteínas citoplasmáticas de *S. aureus* COL mudou na presença de bovicina HC5 em concentração subinibitória. A síntese de diversas proteínas envolvidas na biossíntese de aminoácidos, principalmente produtos do operon *ilv-leu*, e do

metabolismo de DNA, como a enzima DNA polimerase I (PolA), diminuiu após as células de *S. aureus* serem expostas à bovicina HC5. Proteínas envolvidas no catabolismo (ciclo do ácido tricarboxílico) e chaperonas (DnaK, GrpE, GroEL, ClpP e ClpB) apresentaram síntese aumentada. A síntese de importantes reguladores transcricionais (CodY e CcpA) também aumentou na presença da bacteriocina. Estes resultados indicam que bovicina HC5 e nisina podem diminuir a adesão bacteriana a superfícies poliméricas e, consequentemente, diminuir a formação de biofilmes, pela redução das características hidrofóbicas das superfícies da célula e do poliestireno. O estresse causado por bovicina HC5 na membrana perturba a homeostasia celular levando *S. aureus* a paralisar seu crescimento, reduzir a síntese de proteínas e, ao mesmo tempo, aumentar a síntese de chaperonas e enzimas envolvidas no catabolismo de eficiência energética na tentativa de recuperar sua energia e manter a homeostase. Estas informações são importantes para a compreensão da ação das bacteriocinas na inibição da adesão bacteriana e também para entender os mecanismos de resposta visando revelar novos alvos potenciais para o controle de patógenos.

ABSTRACT

PIMENTEL FILHO, Natan de Jesus, D.Sc., Universidade Federal de Viçosa, February, 2014. **Effect of bacteriocins on the adhesion and protein expression profiling of *Staphylococcus aureus***. Adviser: Maria Cristina Dantas Vanetti. Co-adviser: Hilário Cuquetto Mantovani.

Staphylococcus aureus is a human opportunistic pathogen responsible for a broad spectrum of infections ranging from food poisoning and superficial skin infections to more serious diseases such as endocarditis, pneumonia, meningitis, sepsis, and toxic shock syndrome. *S. aureus* is able to adhere to surfaces and form biofilms and, in the food industry, these biofilms may lead to cross-contamination causing risks for food safety. This study aims to evaluate the effect of subinhibitory concentrations of the bacteriocins bovicin HC5 and nisin on the adhesion of *S. aureus* to polystyrene surface, and by using proteomic approach, the stress response of this pathogen to bovicin HC5. Subinhibitory dosages of the bacteriocins, which did not prevent bacterial growth, reduced cell adhesion probably due to changes in the hydrophobicity of the bacterial cell and polystyrene surfaces. Both surfaces became more hydrophilic and the free energy of adhesion between bacteria and polystyrene surface was thermodynamically unfavorable after treatment with bovicin HC5 and nisin. The transcript levels of genes related to adhesion and quorum sensing mechanism were assessed by RT-qPCR approach, revealing that both bacteriocins affected the expression of *icaD*, *fnbA*, and *clfB*, biofilm associated genes, and also *rnaIII*, involved in quorum sensing system. Expression of *icaD* and *fnbA* was up-regulated by bovicin HC5 and nisin. While *clfb* expression decreased in the presence of bovicin HC5, the transcript levels of rnaIII enhanced in the presence of nisin. The synthesis profile of cytoplasmatic proteins of *S. aureus* COL changed in the presence of subinhibitory concentration of bovicin HC5. Synthesis of several proteins involved in amino acids biosynthesis, mainly products of *ilv-leu* operon, and DNA metabolism, such as DNA polymerase I (PolA), decreased

following bovicin HC5 treatment while proteins involved in catabolism (TCA cycle) and chaperones (DnaK, GrpE, GroEL, ClpP, and ClpB) were over-expressed. The synthesis of important regulators (CodY and CcpA) also increased in the presence of the bacteriocin. These results indicate that bovicin HC5 and nisin can reduce bacterial adhesion in polymeric surface, and consequently biofilm establishment, by reducing hydrophobic characteristics of the cell surface and also polystyrene surface. Stress caused by bovicin HC5 in the cell membrane disturbs cell homeostasis leading *S. aureus* to stop growth, reduce protein synthesis and, at the same time, enhance synthesis of chaperones and enzymes involved in energy-efficient catabolism in attempt to restore its energy and maintain cell homeostasis. These are important information to understand the action of bacteriocins on bacterial adhesion inhibition and also to understand response mechanisms in attempt to reveal new potential targets for pathogen control.

GENERAL INTRODUCTION

Staphylococcus aureus is an opportunistic human pathogen that causes a range of illnesses from mild skin infections to life-threatening diseases. Its pathogenicity results from a combination of toxin-mediated virulence, invasiveness and antibiotic resistance.

In the food industry, *S. aureus* causes great concern mainly due to the ability to adhere and form highly resistant biofilms on surfaces of food processing representing a high-risk source of cross-contamination, and once contaminating food, their growth can result in the production of enterotoxins, the causative agent of frequent food poisonings.

Bacterial adhesion and, consequently biofilm formation, depends on the physical-chemical properties of the bacterial surface and food contact surfaces and also the characteristics of growth medium and other environmental conditions. These properties include hydrophobicity, electrical charge and roughness of biotic and abiotic surfaces.

Biofilms are difficult to eradicate due to their resistant characteristic. However, conventional cleaning and disinfection regimens may also contribute to inefficient biofilm control and to the dissemination of resistance. Thus, new control strategies are constantly emerging with main incidence in the use of enzymes, phages, interspecies interactions, and antimicrobial molecules from microbial origin.

Antimicrobial peptides, such as bacteriocins, can be a powerful strategy to prevent cell adhesion. Bacteriocins are bacterially produced peptides or proteins that show bacteriostatic and/or bactericidal activity against other bacteria, and have been widely studied because of their broad bactericidal spectrum with possible application in a wide variety of foods. It has been suggested that bacteriocins are valuable alternatives to antibiotics because they have many properties (e.g., significant potency, high stability, low toxicity, and broad spectra of activity) that make them suitable for many applications. These molecules are especially active against gram-positive bacteria and

can exert the antimicrobial effect through different mechanisms at which the cell envelope is generally the target.

Bacteriocins at subinhibitory concentrations interfere with the cell homeostasis causing a stress response leading the bacteria to regulate a number of mechanisms in an attempt to resist and survive to the action of these antimicrobial agents, especially in damage to the membrane by pore formation. Bovicin HC5 and nisin, lantibiotics (class I bacteriocins), share a similar mode of action using lipid II, the essential cell wall precursor, as docking molecule and forming pores at sensitive membranes.

Gel-based proteomic is the most popular and versatile method of global and differential protein separation and quantification. It is an excellent approach to visualize the pattern and the level of the proteins expressed under defined conditions. Conventional two-dimensional electrophoresis (2-DE) in combination with advanced mass spectrometric techniques facilitate the rapid characterization of thousands of proteins in a single polyacrylamide gel, providing a wide range of information on cell physiology, metabolism and stress responses.

This study aimed to better understand the interference of subinhibitory concentrations of the bacteriocins bovicin HC5 and nisin on the adhesion of *S. aureus* to polystyrene surface and by proteomic approach to evaluate the stress response of this pathogen to bovicin HC5.

CHAPTER 1

BOVICIN HC5 AND NISIN REDUCE *Staphylococcus aureus* ADHESION TO POLYSTYRENE BY CHANGES IN THE HYDROPHOBICITY PROFILE AND GIBBS FREE ENERGY OF ADHESION

ABSTRACT

Staphylococcus aureus is an opportunistic pathogen very often multidrug-resistant that not only causes a variety of human diseases, but also is able to survive on biotic and abiotic surfaces through biofilm communities. The best way to inhibit biofilm establishment is preventing cell adhesion. In the present study, subinhibitory concentrations of the bacteriocins bovicin HC5 and nisin were tested for their capability to interfere with the adhesion of *S. aureus* to polystyrene. Subinhibitory dosages of the bacteriocins reduced cell adhesion probably due to changes in the hydrophobicity of the bacterial cell and polystyrene surfaces. The surfaces became more hydrophilic and the free energy of adhesion ($\Delta G_{adhesion}$) between bacteria and polystyrene surface was unfavorable after treatment with bovicin HC5 and nisin. The transcript level of selected genes was assessed by RT-qPCR approach, revealing that the bacteriocins affected the expression of some important biofilm associated genes (*icaD*, *fnbA*, and *clfB*) and also *rnaIII*, involved in the quorum sensing mechanism. The conditioning of food-contact surfaces with bacteriocins can be an innovative and powerful strategy to prevent biofilm in the food industry. The results are relevant for food safety as they indicate that bovicin HC5 and nisin can inhibit bacterial adhesion and consequently biofilm establishment, since cell adhesion precedes biofilm formation.

Key-words: Food-contact surface, hydrophobicity, foodborne pathogen.

1. INTRODUCTION

Staphylococcus aureus is a common human pathogen responsible for food-borne intoxications worldwide, caused by the ingestion of food containing staphylococcal heat-stable enterotoxins (Le Loir et al., 2003). Because *S. aureus* does not compete well with indigenous microbiota in raw foods, contamination is mainly associated with improper handling of cooked or processed foods, followed by storage under conditions which allow growth of *S. aureus* and production of the enterotoxin (Argudín et al., 2010). Another pathway leading to cross contamination of foods is the capability of some strains to develop biofilm on food-processing surfaces (Vázquez-Sánchez et al., 2013).

Surface-associated microbial biofilm allow bacteria to better resist unfavorable environmental conditions, including the application of external stress stimuli, such as desiccation, UV light, and treatment with antimicrobial and sanitizing agents, due to diffusion of antimicrobials, metabolic activity of biofilm cells, and/or phenotypic variability within the biofilm (Herrera et al., 2007; Parsek and Fuqua, 2004).

Biofilm formation depends on the characteristics of the surface, the bacterial cell, the growth medium, the optical density and other environmental conditions (Donlan and Costerton, 2002). There are several mechanisms by which microbial species are able to come into closer contact with a surface, attach firmly to it, promote cell–cell interactions and grow as a complex structure (Bryers and Ratner, 2004). Adhesion is the initial step in biofilm formation which is mediated mainly by non-specific long-range attractive Lifshitz-van der Waals forces, electrostatic, acid-base, and hydrophobic interaction forces (Busscher et al., 2010; Chung et al., 2014). Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication, and exopolysaccharide production are important for biofilm formation and development (Hori and Matsumoto, 2010; Simões et al., 2010).

Stainless steel is the most frequently used material for food-processing equipments. Polymers are another food-contact surface material widely used in food industry, mainly for packaging. The most commonly used plastics in packaging industry are based on petro chemical products such as polyethylene terephthalate, poly vinyl chloride, polyethylene, polypropylene, polystyrene, and polyamide (Mahalik and Nambiar, 2010). Surface properties, such as hydrophobicity, roughness, and a predisposition to protein adsorption are recognized as important for attachment of

microorganisms to surfaces and the subsequent biofilm development (Araújo et al., 2009; Palmer et al., 2007). The hydrophobicity of the surfaces contributes to the water removal. If two surfaces are hydrophobic, it is easier to eliminate the water layer, due to the molecules of surfaces have less attraction by water molecules and higher interaction with itself (Araújo et al., 2009).

In staphylococcal species, the development of biofilm is mainly facilitated by polysaccharide intercellular adhesion (PIA), a glycan of β-1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl residues of which 15 % are non-*N*-acetylated (Rohde et al., 2010). The polysaccharide is synthesized by enzymes encoded by the *icaADBC* operon. The IcaA, IcaC, and IcaD proteins are located in the membrane fraction; *icaB* gene is mainly found in the culture supernatant and deacetylates PIA when it is localized on the cell surface (Gerke et al., 1998). Co-expression of *icaA* and *icaD* increases N-acetylglucosaminyl transferase activity and slime production (Arciola et al., 2006; Atshan et al., 2013).

Intercellular signaling, often referred to quorum sensing, is reported to be involved in biofilm development (Yarwood et al., 2004). *S. aureus* quorum sensing system is encoded by the accessory gene regulator (*agr*) locus, a global regulatory network of virulence factors including biofilm development. The *agr* chromosomal locus has two promoters, P2 and P3, that control the expression of the divergent RNAII and RNAIII transcripts, respectively. RNAII encodes the core components of the *agr* system, including the AgrB (transmembrane endopeptidase), AgrD (precursor of the autoinducing peptide), AgrC (histidine kinase), and AgrA (regulator) proteins. The RNAIII transcript is itself the main effector for the *agr* system and thereby coordinates the upregulation of secreted virulence factors and the downregulation of cell surface proteins involved in the bacterial attachment (Novick and Geisinger, 2008; Pang et al., 2010).

Bacteriocins, ribosomally synthesized peptides produced by bacteria, have been suggested to inhibit the adhesion of pathogens to abiotic surface (Winkelströter et al., 2011). Many of these substances may be useful as antimicrobial agents for practical applications (Fagundes et al., 2011). Nisin, the most well-studied bacteriocin, is a lantibiotic produced by *Lactococcus lactis* subsp. *lactis* used in over 50 countries as a food preservative. Bovicin HC5 is a bacteriocin with a broad spectrum of action produced by *Streptococcus bovis* HC5, isolated from rumen of cattle (Mantovani et al., 2002). Inhibition of the gram-positive foodborne pathogens *S. aureus*, *Listeria monocytogenes*, *Bacillus cereus*, and *Clostridium perfringens* by the bacteriocins

bovicin HC5 and nisin has been reported (Antolinos et al., 2011; Arques et al., 2008; Mantovani and Russell, 2003; Pimentel-Filho et al., 2013; Pimentel-Filho et al., 2014; Solomakos et al., 2008; Udompijitkul et al., 2011). Bacteriocins with anti-staphylococcal activity, such as bovicin HC5, could open new possibilities to prevent adhesion and thus, to control biofilm formation. Highlighting the potential of antimicrobial peptides against biofilm as a current alternative, recent studies has been reported evaluating the effectiveness of bacteriocins such as nisin, lacticin Q, nukacin ISK-1, and enterocin AS-48 against staphylococcal biofilm (Caballero Goméz et al., 2013; Cabo et al., 2009; Davison et al., 2010; Okuda et al., 2013).

Another interesting strategy to control bacterial adhesion and subsequent biofilm formation is to modify the surface by conditioning or incorporating bacteriocins directly in surfaces which are in direct contact with food. Nostro et al. (2010) incorporated different concentrations of nisin in poly-ethylene-co-vinyl acetate (EVA) films, used for food packing, to evaluate its effect on the biofilm-forming ability of *L. monocytogenes*, *S. aureus* and *Staphylococcus epidermidis.* The results revealed the efficacy of EVA/nisin films in reducing biofilm formation on their surfaces with more evident effect for *S. epidermidis* than *L. monocytogenes* and *S. aureus* strains.

In this work we aimed to evaluate the effect of subinhibitory dosages of the bacteriocins bovicin HC5 and nisin on the adhesion of *S. aureus* to polystyrene surface, determining changes on the surfaces hydrophobicity and free energy of adhesion, and by RT-qPCR approach, investigate the hypothesis that subinhibitory concentration of the bacteriocins affects the expression rate of genes involved in biofilm formation in the evaluated conditions.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

The bacterial strains used in the present study are shown in the Table 1.

The bovicin HC5-producing strain *S. bovis* HC5 was cultivated under anaerobic conditions, at 39°C overnight, in basal medium containing (per liter) 0.292 g K_2HPO_4, 0.292 g KH_2PO_4, 0.48 g $(NH_4)_2SO_4$, 0.48 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.064 g $CaCl_2 \cdot 2H_2O$, 0.5 g cysteine hydrochloride, 4 g Na_2CO_3, 0.1 g trypticase, and 0.5 g yeast extract. Glucose (4 g/l) was added as a carbon source.

S. aureus strains were grown aerobically with vigorous agitation at 37°C in synthetic medium (Gertz et al., 1999).

Table 1. Strains used in this study

Strain	Origen	Reference
S. bovis		
HC5	Isolated from bovine rumen	(Mantovani et al., 2001)
S. aureus		
COL	Isolated from human, MRSA*	(Shafer and Iandolo, 1979)
RN 6911	RN 6390-isogenic *agr* mutant	(Novick et al., 1993)
HG 001	NCTC 8325 derivative, MSSA**	(Herbert et al., 2010)
D4-106.06	Isolated from bovine mastitis	(Poutrel and Lerondelle, 1978)

*MRSA, methicillin-resistant *S. aureus*; MSSA**, methicillin-sensitive *S. aureus.*

2.2 Bacteriocins

Nisin A from *L. lactis* (2.5% nisin, ≥ 1,000 IU mg^{-1}; Sigma-Aldrich, Germany) was resuspended in sterile sodium phosphate buffer (10 mM, pH 7.2) and stored at 7°C until use.

Extracts of bovicin HC5 were prepared as described by Mantovani et al. (2002). Briefly, stationary-phase *S. bovis* HC5 were harvested by centrifugation and the cells were washed in sodium phosphate buffer (10 mM, pH 7.2). The cell pellet was re-suspended in acidic NaCl (100 mM, pH 2.0) for 2 h at room temperature. The suspensions were centrifuged to remove cells and the cell-free supernatant was lyophilized. The lyophilized material was suspended in sterile sodium phosphate buffer (10 mM, pH 2.0). Purification of bovicin HC5 was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a semi-preparative column (Shimadzu C18, Japan; length, 150 mm; inner diameter, 4.6 mm; particle size, 5 µm). The column was equilibrated with buffer A (0.1% trifluoroacetic acid [TFA] in water), and the peptide was eluted using a linear gradient of 35 to 50% buffer B (80% acetonitrile, 0.1% TFA in water) at 22°C and at a flow rate of 1 ml/min. The absorbance was monitored at 214 and 280 nm, and the eluted fraction corresponding to pure bovicin HC5 was lyophilized (Paiva et al., 2011). Bacteriocin stock solutions were stored at 7°C in sterile sodium phosphate buffer (10 mM, pH 7.2) until use.

Bovicin HC5 concentration was determined using a ninhydrin assay (Starcher, 2001). Briefly, bovicin HC5 extract was mixed with equal volume of hydrogen chloride (12 N) for acid-hydrolysis at 100ºC for 24 h. Ninhydrin (1 g) was dissolved in 37.5 ml of ethylene glycol and 12.5 ml of 4 N sodium acetate buffer (pH 5.5). Stannous chloride solution (1.25 ml) freshly prepared by dissolving 50 mg of stannous chloride in 500 µl of ethylene glycol, was added to the ninhydrin-reagent. The acid-hydrolyzed sample

was diluted 100 folds with distilled water. To the tubes containing 200 µl of distilled water, 200 µl of the diluted samples and 600 µl of ninhydrin-reagent were added. After incubation at 100ºC for 10 min in the dark, the absorbance was measured at 575 nm. The standard curve was constructed with bovine serum albumin (Sigma-Aldrich, Germany), in a concentration of 0.25-10 µg μl^{-1} in distilled water.

2.3 Susceptibility to the bacteriocins and adhesion testing

In order to determine the Minimal Inhibitory Concentration (MIC) of bovicin HC5 and nisin, 200 µl of synthetic medium supplemented with increasing bovicin HC5 and nisin concentrations (from 2.0 to 0.2 µM) were transferred to 96-wells microtiter plates, and inoculated with 5 x 10^5 cfu ml^{-1} of exponentially growing *S. aureus* cells (optical density at 500 nm [OD_{500}], 0.5) previously propagated in the same medium without bacteriocins. The minimal concentration that prevented turbidity of the medium after 18 h incubation at 37ºC was designated as the bovicin HC5 and nisin MIC.

To evaluate the effect of bovicin HC5 and nisin on staphylococcal adhesion, assays were carried out using the same experimental design as previously described for MIC experiments. After 18 h of incubation, the culture supernatant was discarded, and the surface-attached cells were stained with 200 µl of 0.1% (w/v) crystal violet for 30 min. Subsequently, the crystal violet was removed and the plate was washed three times with water. After air drying for 15 min at 40ºC, the attached cells were determined at 590 nm with the microtiter plate reader (Biotek, Germany) by addition of 200 µl of 95% (v/v) ethanol.

MIC and adhesion experiments were conducted in three biological replicates and three technical replicates. Data were expressed as the ratio between the absorbance of violet crystal extract (adhered cells) and the optical density of total cells (planktonic and adhered cells) (Viana et al., 2009).

2.4 Contact angle measurement

2.4.1 Surface

Polystyrene coupons (20 mm x 10 mm x 1.00 mm) were first cleaned by washing with liquid neutral detergent and water, following by rinsing with distilled water and then immersion in 70% ethyl alcohol for 1 h to remove fat. Subsequently, they were rinsed with distilled water and air dried under UV light. The cleaned and sanitized coupons were pre-conditioning for 18 h at 37ºC by immerging in synthetic medium without bacteriocins or synthetic medium with 0.4 µM of bovicin HC5 or nisin.

The contact angles between the surface and the ultra-pure water, formamide (LGC Bio, Brazil) and α-bromonaphtalene (Merck, Germany) were determined using a goniometer (Kruss, Germany). Contact angle measurements of 2.0 µl drop were taken each second for 30 s for all liquids. Experiments were conducted in three biological replicates and three technical replicates.

2.4.2 Microorganism

Contact angles of *S. aureus* COL surface were determined on a layer of vegetative cells (Busscher et al., 1984). First, pre-warmed medium at 37ºC was inoculated with cells from an overnight culture to an initial OD_{500} of 0.1 and monitored by measuring the OD until the culture reached an OD_{500} of 0.5. At that time, the culture was exposed to 0.4 µM of bovicin HC5 or nisin followed by statically incubation at 37ºC for 18 h. Growth in medium without bacteriocins was performed as a control.

Later, the suspension was centrifuged at 4,000 x *g* at 4°C for 10 min and then washed three times in 0.1 M phosphate-buffered saline (PBS). The pellet was resuspended in the same buffer and then filtered using acetate cellulose membrane (0.45 µm pore size, 47 mm diameter). During the filtration, 30 ml of ultra-pure water was added.

To standardize the moisture content, the membranes were transferred into Petri dishes containing 1% (w/v) agar and 10% (v/v) glycerol. The membranes were cut to determine the contact angle with the three different polarities liquids.

2.4.3 Determination of the total interfacial tension (γ_s^{tot})

The total interfacial tension was determined by the sum of the apolar and polar components of the respective surfaces (Equation 1):

$$\gamma_l^{TOT}(1+\cos\theta) = 2\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + 2\sqrt{\gamma_s^{-}\gamma_l^{+}} + 2\sqrt{\gamma_s^{+}\gamma_l^{-}} \qquad (1)$$

where γ_l^{TOT} is the total interfacial tension of the liquid; γ^{LW} is the interfacial tension of the interactions of the Lifshitz-van der Waals forces; γ^{+} is the interfacial tension of the electron acceptor component of the acid-base component; γ^{-} is the interfacial tension of the electron donor component of the acid-base component, θ is the contact angle, and *s* and *l* indicate surface and liquid, respectively (Van Oss and Giese, 1995).

The three components of the interfacial tension of the surfaces were determined from the contact angles obtained from three liquids with different polarities, whose interfacial tensions are known, as shown in Table 2.

Table 2. Components of the interfacial tensions of the substances at 25ºC

Substances	Interfacial tension (mJ/m^2)			
	γ_l^{TOT}	γ_l^{LW}	γ_l^{+}	γ_l^{-}
α-Bromonaphthalene	44.4	44.4	0.0	0.0
Water	72.8	21.8	25.5	25.5
Formamide	58.0	39.0	2.28	39.6

The interfacial tension is the result of the sum of the two components (γ_s^{LW} and γ_s^{AB}):

$$\gamma_s^{LW} = 11.1(1+\cos\theta_B)^2 \quad (2)$$

$$\gamma_s^{AB} = 2\sqrt{\gamma_s^{+}\gamma_s^{-}} \quad (3)$$

$$\gamma_s^{tot} = \gamma_s^{LW} + \gamma_s^{AB} \quad (4)$$

where γ_s^{LW} is the interfacial tension of the interactions of the Lifshitz-van der Waals forces; θ_B is the contact angle obtained with α-bromonaphthalene; γ_s^{AB} is the polar component of the Lewis acid-base interaction; γ_s^{+} is the interfacial tension of the electron acceptor component of the acid-base component; γ_s^{-} is the interfacial tension of the electron donor component of the acid-base component and γ_s^{tot} is the total interfacial tension of the surface.

2.4.4 Free energy of interaction (ΔG_{sws}^{TOT})

The total free energy of interaction among molecules of the surface (s) immersed in water (w) was determined by the sum of the apolar and polar free energies of interaction, ΔG_{sws}^{LW} and ΔG_{sws}^{AB}, respectively:

$$\Delta G_{sws}^{tot} = \Delta G_{sws}^{LW} + \Delta G_{sws}^{AB} \quad (5)$$

$$\Delta G_{sws}^{LW} = -2.\sqrt{\gamma_s^{LW} - \gamma_w^{LW}} \quad (6)$$

$$\Delta G_{sws}^{AB} = -4\left(\sqrt{\gamma_s^{+}\gamma_s^{-}} + \sqrt{\gamma_w^{+}\gamma_w^{-}} - \sqrt{\gamma_s^{+}\gamma_w^{-}} - \sqrt{\gamma_w^{+}\gamma_s^{-}}\right) \quad (7)$$

2.4.5 Determination of the total free energy of adhesion ($\Delta G_{adhesion}$)

Using the values of the components of the interfacial tensions, it is possible to determine the $\Delta G_{adhesion}$ between two surfaces (microbial cells (b) and polystyrene surface (s)):

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (8)$$

$$\gamma_{bs}^{LW} = \gamma_{b}^{LW} + \gamma_{s}^{LW} - 2\sqrt{\gamma_{b}^{LW}\gamma_{s}^{LW}} \quad (9)$$

$$\gamma_{bs}^{AB} = 2\left(\sqrt{\gamma_{b}^{+}\gamma_{b}^{-}} + \sqrt{\gamma_{s}^{+}\gamma_{s}^{-}} - \sqrt{\gamma_{b}^{+}\gamma_{s}^{-}} - \sqrt{\gamma_{b}^{-}\gamma_{s}^{+}}\right) \quad (10)$$

When free energy is related to the interfacial tension, $\Delta G_{adhesion}$ can then be represented by the following:

$$\Delta G_{adhesion} = \Delta G_{bls}^{LW} + \Delta G_{bls}^{AB} \quad (11)$$

$$\Delta G_{bls}^{LW} = \gamma_{bs}^{LW} - \gamma_{bl}^{LW} - \gamma_{sl}^{LW} \quad (12)$$

$$\Delta G_{bls}^{AB} = \gamma_{bs}^{AB} - \gamma_{bl}^{AB} - \gamma_{sl}^{AB} \quad (13)$$

where γ_{bs} is the interfacial tension between the bacterial surfaces and the adhesion surface; γ_{bl} is the interfacial tension between the bacterial surfaces and the liquid; and γ_{sl} is the interfacial tension between the adhesion surfaces and the liquid.

The $\Delta G_{adhesion}$ values allow for evaluation of the thermodynamics of the adhesion process: if $\Delta G_{adhesion} < 0$, the process is favorable; if $\Delta G_{adhesion} > 0$, the process is unfavorable.

2.5. Impact of bovicin HC5 and nisin on biofilm-related gene expression in *S. aureus*

2.5.1 Sample preparation and total RNA extraction

Expression of genes related to adhesion and biofilm formation *icaD*, *fnbA*, *clfB*, and *rnaIII* was evaluated after exponentially growing cells of *S. aureus* COL (OD_{500} 0.5) have been exposed to 0.4 µM bovicin HC5 or nisin in synthetic medium for 18 h, statically at 37ºC. Untreated cells were also evaluated after 18 h of growth in synthetic medium.

Bacterial cells were collected by centrifugation for 5 min at 6,000 x *g*. Total RNA was obtained by phenol-chloroform extraction (TRIzol®, Invitrogen, USA) according to the manufacturer's instructions after a 40 min pre-treatment of the cells with 0.8 µg/µl of lysozyme and 0.00002 U/µl of lysostaphin in 300 µl of TE buffer. Purified RNA was eluted in 30 µl of Ultrapure™ distilled water RNase/DNase-free (Invitrogen, USA) and stored at -20ºC. The concentration and purity of the RNA was evaluated by spectrometry at 260 nm and 280 nm. The integrity of the RNA was confirmed by 1% (w/v) agarose gel electrophoresis.

2.5.2 cDNA synthesis and RT-qPCR

Aliquots of 2 µg of total RNA were treated with RQ1 RNase-Free DNase (Promega, USA) for DNA elimination according to the manufacturer's instructions. cDNA synthesis was performed using Improm-II Reverse Transcription System kit (Promega, USA) following the manufacturer's recommendations. The mixture was incubated at 25ºC for 5 min, 42ºC for 2 h and 70ºC for 15 min.

The relative mRNA levels of biofilm-related genes were analyzed by quantitative real-time polymerase chain reaction (RT-qPCR) using SYBR® Green PCR-Master Mix (Applied Biosystems, USA) in 25 µl reaction volumes containing 1 µl of cDNA template, 1 µl of each primer at 0.5 µM (Table 3), 12.5 µl of SYBR® Green PCR-Master Mix and 9.5 µl of Ultrapure™ distilled water RNase/DNase-free and the following conditions: 50ºC for 2 min, 95ºC for 10 min, 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Abundance of each specific mRNA was calculated relative to the expression of the housekeeping gene DNA gyrase, B subunit (*gyrB*) based on $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The amplifications were performed in 96-well PCR plates using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The fluorescence data for each sample were converted to Cycle threshold (Ct) values using the software Bio-Rad CFX Manager 2.0.

The experiment was performed in two biological replicates and three technical replicates.

Table 3. Sequences of primers used for RT-qPCR

Gene	Nucleotide sequence of primers (5'-3')	Reference
icaD	1-GGGTGGATCCTTAGTGTTACAATTTT 2-TGACTTTTTGGTAATTCAAGGTTGTC	(Korem et al., 2010)
fnbA	1-CGACACAACCTCAAGACAATAGCGG 2-CGTGGCTTACTTTCTGATGCCGTTC	(Ster et al., 2005)
clfB	1-AATGCCATCATTGCACCAAA 2-CACAAAACTGATGCTTTACCAGAAA	(Korem et al., 2010)
rnaIII	1- AGTCACCGATTGTTGAAATGATATCT 2- AGGAAGGAGTGATTTCAATGGC	(Pang et al., 2010)
gyrB	1-ATCGGTGGCGACTTTGATCTA 2-CCACATCGGCATCAGTCATAA	(Korem et al., 2010)

3. RESULTS

3.1 Inhibition of *S. aureus* by bovicin HC5 and nisin

The MICs of the bacteriocins bovicin HC5 and nisin on four different strains of *S. aureus* were investigated in synthetic medium and the results are shown in the Table 4. The MIC values average for bovicin HC5 and nisin was 1.05 ± 0.90 and 0.90 ± 0.26, respectively.

Table 4. Minimal Inhibitory Concentration of bovicin HC5 and nisin on *S. aureus* strains

	Bacteriocins (µM)	
***S. aureus* strains**	**Bovicin HC5**	**Nisin**
COL	1.2	1.0
RN 6911	1.2	1.2
HG 001	0.8	0.6
D4-106.06	1.0	0.8

Subinhibitory dosages of bovicin HC5 and nisin were tested for their capacity to inhibit adhesion of *S. aureus* in polystyrene 96-well microtiter plates after 18 h of incubation using crystal violet method. Adhesion was estimated by calculating the ratio between the absorbance of violet crystal extract and the optical density of total cells. The presence of the bacteriocins clearly reduced adhesion of the strains COL and RN 6911 to polystyrene surface (Figure 1), and the effect of bovicin HC5 and nisin seemed to be similar for COL strain. While only bovicin HC5 was able to reduce adhesion of *S. aureus* HG 001, only nisin at the highest concentration evaluated reduced adhesion of *S. aureus* D4-106.06 to polystyrene.

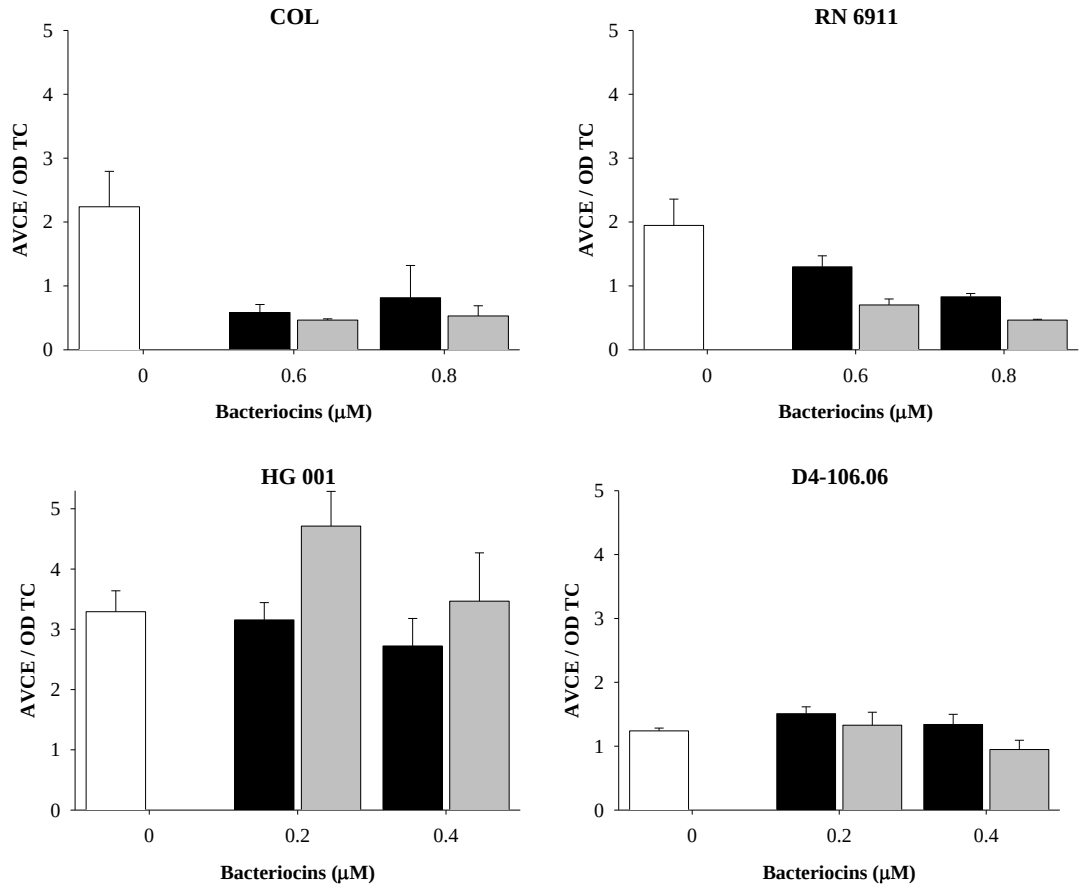

Figure 1. Ratio between the absorbance of violet crystal extract (AVCE) and the optical density of total cells (OD TC) of *S. aureus* following bovicin HC5 or nisin treatment. *S. aureus* COL, RN 6911, HG001, and D4-106.06 were cultivated in synthetic medium for 18h in the presence of different concentration of bovicin HC5 (black bars) and nisin (grey bars). Untreated control (white bars) is shown.

3.2 Contact angle and total free energy of interaction (ΔG_{sws}^{TOT})

As we observed that subinhibitory dosages of bovicin HC5 and nisin were more effective on adhesion reduction than growth reduction, COL strain was selected to investigate if the presence of bovicin HC5 and nisin in low concentration could change the hydrophobicity of the bacterial surface and also the polystyrene surface and thus, interfere with the adhesion process.

The measurement of the water contact angle (Θ_W) with the polystyrene surface exposed for 18 h to synthetic medium without bacteriocins was greater than 65º, indicating a hydrophobic profile according to the classification system (Vogler, 1998). The presence of the bacteriocins in the synthetic medium drastically changed the hydrophobicity of the polystyrene surface, since Θ_W with the surface was much lower than 65º (Table 5). *S. aureus* COL cellular surface, hydrophilic in the absence of

bacteriocins, remained hydrophilic with a small decrease in the contact angle when bacteria grew in the presence of 0.4 µM of bovicin HC5 or nisin.

The ΔG_{sws}^{TOT} values are considered a quantitative criterion for hydrophobicity evaluation. According to the calculations, polystyrene surface treated with synthetic medium without bacteriocins was classified as hydrophobic ($\Delta G_{sws}^{TOT} < 0$) (Table 5). The presence of bovicin HC5 and nisin decreased the hydrophobicity of the polystyrene surface. The bacterial surface, hydrophilic, remained hydrophilic ($\Delta G_{sws}^{TOT} > 0$) even after 18 h of exposition to the bacteriocins (Table 5).

Table 5. Averages and standard deviation of contact angles measurements with water (Θ_W), formamid (Θ_F) and α-bromonaphtalene (Θ_B), and total free energy of interaction ($\Delta \boldsymbol{G}_{\mathbf{sws}}^{\mathbf{TOT}}$) of *S. aureus* COL and polystyrene surface (PS) treated with bovicin HC5 and nisin

Surface/Bacteria	Contact angles (º)[a]			ΔG_{sws}^{TOT} (mJ/m^2)
	Θ_W	Θ_F	Θ_B	
PS + Synthetic medium	82.4 ± 4.4	66.2 ± 5.5	22.9 ± 1.4	-63.7
PS + Synthetic medium + 0.4 µM bovicin HC5	33.4 ± 7.9	30.2 ± 2.9	32.7 ± 4.8	22.4
PS + Synthetic medium + 0.4 µM nisin	40.5 ± 1.3	37.0 ± 0.8	40.9 ± 2.7	18.1
S. aureus + Synthetic medium	25.3 ± 2.9	17.7 ± 0.3	45.0 ± 1.5	20.3
S. aureus + Synthetic medium + 0.4 µM bovicin HC5	21.9 ± 3.1	16.6 ± 0.7	45.9 ± 1.9	22.8
S. aureus + Synthetic medium + 0.4 µM nisin	23.5 ± 1.1	25.3 ± 3.8	45.8 ± 2.3	28.4

[a]Average of three repetitions.

3.2.1 Free energy adhesion ($\Delta G_{adhesion}$)

According to thermodynamic theory, adhesion is considered favorable only if the process results in a decrease in total free energy. Thus, the adhesion process was thermodynamically favorable ($\Delta G_{adhesion} < 0$) only when the synthetic medium in contact with the polystyrene surface contained no bacteriocins (Table 6). In the presence of bovicin HC5 or nisin, the adhesion process was considered thermodynamically unfavorable ($\Delta G_{adhesion} > 0$) (Table 6), confirming the previously results that both bacteriocins decreased the adhesion of *S. aureus* COL.

Table 6. Free energy of adhesion (mJ/m^2) between *S. aureus* COL and polystyrene surface treated with bovicin HC5 and nisin

***S. aureus* x Polystyrene Surface**	$\Delta G_{adhesion}$
Synthetic medium	-9.58
Synthetic medium with bovicin HC5	23.30
Synthetic medium with nisin	24.06

3.3 Effect of bovicin HC5 and nisin on the expression of biofilm-related genes in *S. aureus*

After verifying that subinhibitory dosages of bovicin HC5 and nisin reduced the adhesion of *S. aureus* COL to polystyrene microtiter plates been confirmed by decreasing of the hydrophobicity, we investigated the effect of the bacteriocins on the expression of some selected genes related to adhesion and biofilm formation (*icaD*, *fnbA* and *clfB*) and also on the *rnaIII* involved in the regulation by quorum sensing.

Compared to the control without bacteriocins, low concentrations of bovicin HC5 and nisin increased by 2.62 and 5.79 fold, respectively, the expression of *icaD* (Figure 2), one of the most studied genes related to biofilm formation by staphylococci. Nisin exert greater influence on increasing *icaD* expression. Bovicin HC5 and nisin were also able to increase the expression of *fnbA*, fibronectin A, in 1.34 and 1.94 fold, respectively. Again, the effect of nisin was more pronounced than bovicin HC5. The transcription of *clfB*, clumping factor B, did not change when *S. aureus* COL grew for 18 h in the presence of 0.4 µM of nisin. However, bovicin HC5 decreased *clfB* transcription (Figure 2).

The exposition of *S. aureus* COL for 18 h to subinhibitory concentration of bovicin HC5 did not cause significant changes in the transcription profile of *rnaIII*, main effector of *agr* quorum sensing system in *S. aureus*. The presence of nisin in the growth medium enhanced 1.64 fold the expression of *rnaIII*.

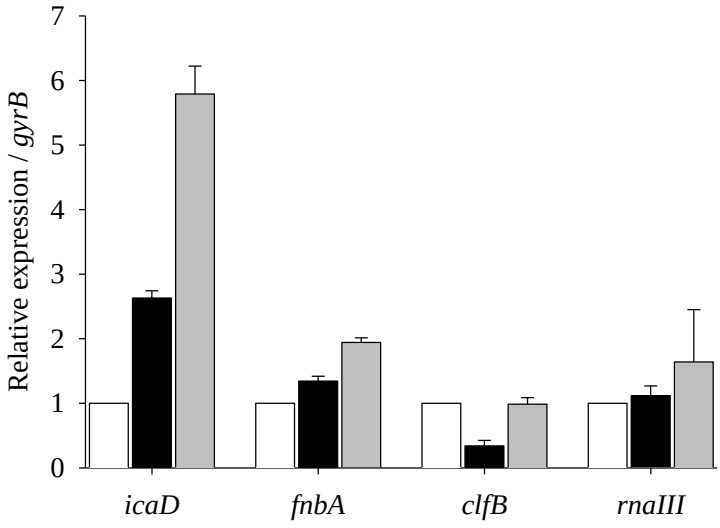

Figure 2. Comparison of the expression profiles of selected biofilm formation-related genes (*icaD*, *fnbA* and *clfB*) and quorum sensing-related (*rnaIII*) in *S. aureus* COL. Cells were grown in synthetic medium and total RNA was extracted from the cells after 18 h of exposition to 0.4 µM of bovicin HC5 (black bars) and nisin (white bars) and the expression profile was analyzed by RT-qPCR. Untreated control (white bars) is shown.

4. DISCUSSION

For long time, *S. aureus* has caused problems in health care. In order to control *S. aureus* growth and biofilm formation various natural substances have been tested. In this work, bovicin HC5 and nisin were tested against different strains of *S. aureus* aiming to determine MIC and also the effects of subinhibitory concentrations on cell adhesion to polystyrene surface. Bovicin HC5 and nisin were effective against all strains of *S. aureus* tested in synthetic medium and similar inhibitory effect was observed for both bacteriocins. The minimum concentrations of bovicin HC5 and nisin able to inhibit the growth of all tested strains was 1.4 and 1.2 µM, respectively. Recently, our group reported that the bacteriocins bovicin HC5 and nisin were effective to inhibit *S. aureus* growth in milk (Pimentel-Filho et al., 2013), and in fresh cheese (Pimentel-Filho et al., 2014). These results reinforce the idea that not only nisin, already authorized as a food additive in many countries, but also bovicin HC5 has potential application as a natural food preservative. Other bacteriocins such as enterocin AS-48 (Ananou et al., 2004; Ananou et al., 2010), reuterin (Arques et al., 2008), epidermicin NI01 (Okuda et al., 2013), and lacticin Q (Sandiford and Upton, 2012) have been tested and were effective against *S. aureus*, even those called drug or multidrug-resistant.

Development of technologies to control microbial adhesion and biofilm formation is desired for food industries. Our study revealed that subinhibitory dosages of bovicin HC5 and nisin reduced the adhesion of the pathogen to the polystyrene frequently used in trays, utensils and food packing. Low dosages of nisin as 25 IU/ml were able to reduce staphylococcal biofilm on polypropylene coupons and were even more effective against planktonic cell of *S. aureus* (Cabo et al., 2009). Bacteriocin-like substances produced by lactobacilli suppressed biofilm formation on polystyrene surface by *S. aureus* and *S. epidermidis* and induced ultrastructural changes leading to their destruction (Sadowska et al., 2010). Davison et al. (2010) verified that nisin accessed the interior of biofilm cells clusters of *S. epidermidis* on glass faster than other antimicrobial agents as chlorine and glutaraldehyde resulting in a rapid and uniform loss of green fluorescence, indicating cell die, without any removal of biofilm. All these results reinforce the idea that bacteriocins can be a potential strategy to prevent adhesion and to control biofilm formation.

Generally, any surface is vulnerable to biofilm development including plastic, glass, metal, wood, and food products but the surface physiochemical properties play an important role in bacterial adhesion (Ferreira et al., 2010). By water contact angle

values, a qualitative criterion, we observed that cell surface of *S. aureus* COL growing in synthetic medium with or without bovicin HC5 and nisin was hydrophilic. Our result was in agreement with reports which found that the *S. aureus* surface is hydrophilic (Hamadi et al., 2005; Kouidhi et al., 2010). Polystyrene surface conditioned for 18 h with synthetic medium was hydrophobic being considered favorable for bacterial adhesion. Evaluating the hydrophobicity of polystyrene surface, Biazar et al. (2011) found a contact angle of 90.1º confirming its hydrophobic character. However, after 18 h of conditioning with synthetic medium added of bovicin HC5 or nisin, the polystyrene surface became hydrophilic (Θ_W < 65º). Our data showed the decrease in the hydrophobicity on treated surfaces indicates one of the reasons for bacterial attachment decrease. Hydrophobicity is an important propriety and represents the wettability of a surface and, in aqueous medium, adhesion is favored between hydrophobic surfaces, which can enter in closer contact by squeezing the water layer between them (Teixeira et al., 2005). After conditioning with surfactant, the polystyrene surface became more hydrophilic (Zeraik and Nitschke, 2010)**.** It has been shown, that *Salmonella* and *Listeria* preferably adhere in a higher numbers to hydrophobic surfaces than the hydrophilic ones (Donlan and Costerton, 2002; Sinde and Carballo, 2000).

In order to predict the ability of the pathogen to adhere to those treated or untreated polystyrene surfaces, and further to form biofilm, the free energy of interaction between the micro-organisms and the surface, when immersed in the same synthetic medium condition with or without bacteriocin, was calculated. Only in the medium without bacteriocins the adhesion process was thermodynamically favorable. Since the total free energy of adhesion was positive, in the medium containing bacteriocins, the adhesion process was considered unfavorable. Teixeira et al. (2005) also demonstrated that adhesion of some isolates of *Pseudomonas aeruginosa* and *Staphylococcus sciuri* was thermodynamically favorable to stainless steel and rubber, which were considered hydrophobic and unfavorable to glass and polymethylacrylate surfaces, classified as hydrophilic.

Although many studies have been performed aiming on a better understanding of biofilm formation by *S. aureus*, the knowledge on the effect of bacteriocins on gene expression is still limited. Our work investigated exposure to bovicin HC5 and nisin was also related to an altered expression of four selected genes involved in biofilm formation by *S. aureus* COL. Interestingly, we found *icaD* over-expressed in the presence of both bovicin HC5 and nisin. In staphylococci, production of polysaccharide intercellular adhesion (PIA) by the enzyme products of the *icaADBC* operon is the best

understood mechanism of biofilm development, making the *ica* genes a potential target for biofilm inhibitors (Oduwole et al., 2010). Similar effect was observed by Nuryastuti et al. (2009) when evaluating subinhibitory concentration of cinnamon oil on the expression of *icaA* in *S. epidermidis*, another important gene of *icaADBC* operon evaluated in studies involving biofilm formation by staphylococcal species. They found that even reducing biofilm formation on polystyrene surface, 0.01% of cinnamon oil enhanced *icaA* expression (Nuryastuti et al., 2009). On the other hand, low dosages of povidone-iodine, a complex of polyvinyl pyrrilidine and triiodine ions widely used as an antiseptic in trauma and orthopaedic surgery, descreased *icaA* expression and, thus, the biofilm forming capacity of *S. aureus* RN4220 (Oduwole et al., 2010). According to the reports in the literature, the expression of the *ica* genes is highly variable and can be induced by variations in the culture conditions, such as an increase in the concentration of sugars or other substances that induce stress (Cho et al., 2002; Oliveira and de Lourdes Cunha, 2010).

Bovicin HC5 and nisin were also able to up-regulate *fnbA*, meanwhile bovicin HC5 slightly reduced *clfB*. As found by us, Rasigade et al. (2011) reported that although subinhibitory concentration of rifampicin reduced bacterial adhesion to human fibronectin, the antibiotic did not affect *fnbA/B* transcription by five *S. aureus* strains. However, low dosages oxacillin, moxifloxacin and linezolid led to the development of a hyper-adhesive phenotype in the fibronectin adhesion assay, increasing also *fnbA/B* expression (Rasigade et al., 2011). Sublethal dosages of tigecycline were investigated on biofilm formation by 16 methicilin-resistant *S. aureus* isolates and the transcriptome analysis revealed that the antibiotic was able to reduced expression of *icaC*, otherwise upregulation of *fnbA* and *clfB*, which encode adhesins which attach to human proteins, was observed (Smith et al., 2010).

Biofilm formation can be induced by conditions that are potentially toxic for bacterial cells, such as high levels of osmolarity, detergents, urea, ethanol, oxidative stress, and the presence of sub-MICs of some antibiotics (Nuryastuti et al., 2009).

No difference on *rnaIII* expression was observed in the presence of bovicin HC5 while nisin slightly enhanced the transcription of *rnaIII*. The *agr* system effector, *rnaIII*, regulates the expression of a large number of target genes, including down-regulating biofilm-related genes as *fnbA* and *clfB* (Xue et al., 2012), it was expected that the bacteriocins would enhance the *rnaIII* transcription and consequently reduce the expression of *fnbA* and *clfB*.

To our knowledge, this is the first study reporting that bacteriocins act changing the hydrophobicity of polystyrene surfaces. It seems that even prepared to adhere, since biofilm appendages such as IcaD and FnbA are already synthesized by planktonic cells, if the free energy of adhesion is not favorable it is difficult to the bacteria to get in close contact with the surface. This is a very interesting finding since to prevent microbial adhesion to food contact surfaces is much more effective than to remove biofilm already established. Thus, bovicin HC5 and nisin appear as a potential alternative to inhibit the initial step of biofilm formation.

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CHAPTER 2

PROTEIN EXPRESSION PROFILING OF *Staphylococcus aureus* IN RESPONSE TO BOVICIN HC5

1. INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen very often multidrug-resistant involved in both hospital and community-associated diseases worldwide (Rivera et al., 2012). *S. aureus* causes an array of infectious syndromes, ranging from localized skin lesions and tissue damage to systemic infections, such as pneumonia, endocarditis, and exotoxin syndromes (Lowy, 1998). This diversity of *S. aureus*-associated diseases results from its ability to adapt efficiently to varying environmental conditions, including the production of several secreted and cell wall-associated virulence factors (Majerczyk et al., 2008). Because of the emerging resistance to various antimicrobial agents, *S. aureus* have become ever more difficult to control.

The development of new therapeutic strategies to overcome *S. aureus* infections is thus the ambitious goal for the near future (Kohler et al., 2008). One option that cannot be ignored is a group of antimicrobial peptides known as bacteriocins. These are small, bacterially produced, ribosomally synthesized peptides that are active against other bacteria. The producers are immune to their own bacteriocins, a property that is mediated by specific immunity proteins (Cotter et al., 2005). Many bacteriocins have a high specific activity against clinical targets (including antibiotic-resistant strains), have mechanisms of action that are distinct from current chemotherapeutic products and, given their proteinaceous nature, are amenable to gene-based peptide engineering (Cotter et al., 2013).

Lantibiotics, the most common bacteriocins and produced by lactic acid bacteria, are small peptides (<5 kDa) containing the unusual amino acids lanthionine, α-

methyllanthionine, dehydroalanine, and dehydrobutyrine (Chen and Hoover, 2003). Bovicin HC5 is a lantibiotic produced by *Streptococcus bovis* HC5 and first described by Mantovani et al. (2002). This bacteriocin exhibits a broad antimicrobial spectrum including foodborne pathogens and clinical isolates (Mantovani and Russell, 2003; Pimentel-Filho et al., 2013; Prudêncio et al., 2014) and also spoilage bacteria (Carvalho et al., 2007; Carvalho et al., 2008).

The mode of action of bovicin HC5 is similar to nisin, the most studied bacteriocin and the only one approved for food applications (Paiva et al., 2011; Ross et al., 2002). Their amino acid composition, amphipathicity, cationic charge and size allow them to attach to and insert into membrane. The mechanism of action of bovicin HC5 was partially elucidated and, such as nisin, it seems to be based on the specific interaction with lipid II molecule, leading to inhibition of the bacterial cell wall synthesis and eventually to pore-formation (Paiva et al., 2011). Of note, the pore-forming ability of bovicin HC5 seems to be dependent on membrane thickness and composition and determines the inhibitory spectra of these lantibiotic (Paiva et al., 2011).

Global protein expression profiling is an excellent approach to visualize the pattern and the level of the proteins expressed under defined conditions (Wolf et al., 2008). Conventional two-dimensional electrophoresis (2-DE) in combination with advanced mass spectrometric techniques has facilitated the rapid characterization of thousands of proteins in a single polyacrylamide gel, providing a wide range of information on cell physiology, metabolism, and stress responses (Chevalier, 2010). Most of the metabolic pathways are covered by gel-based proteomics offering the possibility to reconstruct the active metabolism at a proteome-wide scale and to analyze the regulation of entire metabolic pathways (Hecker et al., 2010). By labeling newly synthesized proteins with L-[^{35}S]-methionine before and after stress or starvation, protein synthesis patterns can be visualized by 2-DE, allowing the identification of marker proteins whose synthesis is induced or repressed under stress or starvation (Wolf et al., 2008). These representative marker proteins for a single stress condition constitute a so-called proteomic signature.

In this work we investigate the response of *S. aureus* COL in the presence or absence of subinhibitory concentrations of bovicin HC5 using gel-based proteomics aiming to obtain a more comprehensive understanding on the physiological processes involved in the adaptation and survival of *S. aureus* stressed by the bacteriocin.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

S. aureus COL was grown aerobically at 37°C in synthetic medium (Gertz et al., 1999) and 150 rpm. *Streptococcus bovis* HC5, bovicin HC5 producer strain, was cultivated under anaerobic conditions, at 39°C for 18 h, in basal medium containing (per liter) 0.292 g K_2HPO_4, 0.292 g KH_2PO_4, 0.48 g $(NH_4)_2SO_4$, 0.48 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.064 g $CaCl_2 \cdot 2H_2O$, 0.5 g cysteine hydrochloride, 4 g Na_2CO_3, 0.1 g trypticase, and 0.5 g yeast extract. Glucose (4 g l^{-1}) was added as a carbon source.

For bovicin HC5 labeling experiments, pre-warmed synthetic medium at 37ºC was inoculated with cells from an overnight culture to an initial OD_{500} of 0.075 and monitored each hour by measuring the OD until the culture reached an OD_{500} of 0.5. Then, the culture was split in three parts, and two of them were transferred to pre-warmed Erlenmeyer flasks and treated with 1.0 and 2.0 µM bovicin HC5, while the untreated remaining culture served as a control. The proteome analysis of stressed cells was performed after exposure to bovicin HC5 at concentration of 2.0 µM, proved to reduce the growth rate of *S. aureus* COL by 50% when compared with untreated control.

2.2 Bacteriocins preparation

Extracts of bovicin HC5 were prepared as described by Mantovani et al. (2002). Purification of bovicin HC5 was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a semi preparative column (Shimadzu C18, Japan) (Paiva et al., 2011). Bacteriocin stock solutions were stored at 7°C in sterile sodium phosphate buffer (10 mM, pH 7.2) until use. Bovicin HC5 concentration was determined using a ninhydrin assay (Starcher, 2001).

2.3 L-[^{35}S]methionine labeling and preparation of pulse-labeled protein extracts

In order to investigate changes in the pattern of proteins newly synthesized after the addition of 2.0 µM bovicin HC5, the proteins were pulse-labeled with L-[^{35}S]methionine as described previously (Engelmann and Hecker, 2008). At time points 10, 20, and 40 min after bovicin HC5 treatment, 338 µCi of L-[^{35}S]methionine was added to 15 ml of the respective cell culture. As a control, cells of an untreated culture were pulse-labeled at time point zero (exponential growth at an OD_{500} of 0.5, immediately before stress) after the beginning of the stress experiment. The uptake and

incorporation of radioactively labeled methionine in newly synthesized proteins always lasted exactly 5 min. Protein synthesis was blocked by the addition of 1 ml stop solution (3 mM chloramphenicol, 10 mM unlabeled L-methionine). Cells were harvested on ice, centrifuged for 5 min (9,000 *g* at 4°C), washed twice with ice-cold Tris-EDTA (TE) buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and stored at -20°C.

2.4 Preparation of cytoplasmic proteins for preparative 2D gel electrophoresis

The cells to be submitted to mechanical disruption were resuspended in 400 µl ice-cold TE buffer, and then transferred to screw-cap microtubes (Sarstedt, Nümbrecht, Germany) containing 500 µl of glass beads (diameter 0.01 to 0.1 mm). Cells were disrupted by homogenization using a Ribolyser (Peqlab, Erlangen, Germany) for 30 s at 6.5 m s^{-2} followed by incubation on ice for 5 min and a second disruption in the same condition. In order to remove cell debris and insoluble or aggregated proteins, the lysate was centrifuged for 5 min (21,500 *g* at 4°C). The supernatant was transferred to a new tube, and the centrifugation step was repeated twice for 5 min.

The protein concentration was determined by using Roti-Nanoquant reagent according to the manufacturer's instructions (Roth, Karlsruhe, Germany), and the protein solutions were stored at -20°C.

To measure the incorporation of L-[^{35}S]methionine into proteins, two aliquots of each protein solution (5 µl) was pipetted onto filter paper disks. After drying, they were subjected to 10 min of precipitation with 10% (w/v) trichloroacetic acid (TCA) on ice and washed twice for 10 min with 5% (w/v) TCA on ice and twice for 15 min with 90% (v/v) ethanol at room temperature. After drying, 1 ml toluene was added, and the radioactivity of the dried filter disks was measured as counts per minute on a Packard Tricarb 2900 TR liquid scintillation counter (PerkinElmer, Waltham, USA).

2.5 Analytical and preparative 2-DE

Two-dimensional polyacrylamide gel electrophoresis (2-DE) was performed by using the immobilized pH gradient (IPG) technique. In the first dimension, proteins were separated on IPG strips (Serva, Heidelberg, Germany) in a linear pH range from 4 to 7 (18 cm). For analytical 2-DE, 100 µg of radioactively labeled protein extracts was loaded onto the IPG strips. Analysis by 2-DE were performed as described previously (Buttner et al., 2001). The resulting 2-DE were fixed with 40% (v/v) ethanol and 10% (v/v) acetic acid for 1 h and subsequently stained with Krypton™ protein stain (Thermo Scientific, Rockford, USA) over night after a 5 min wash-step with distilled water

(Michalik et al., 2009). The stained gels were scanned, dried in a vacuum dryer, and fixed onto Whatman paper. Afterward, the dried gels were exposed to storage phosphor screens (GE Healthcare, Germany) and scanned with a Typhoon scanner 9400 (GE Healthcare, Germany) until the signal intensity had reached a maximum value between 90,000 and 100,000. For the identification of protein spots by mass spectrometry (MS), preparative 2-DE was performed. A total amount of 350 µg of protein extracts was loaded onto the IPG strips (Serva, Heidelberg, Germany) in a pH range from 4 to 7 (18 cm). The 2-DE gels obtained were fixed with 40% (v/v) ethanol and 10% (v/v) acetic acid for 1 h and then immediately stained with Krypton (ThermoScientific, Rockford, USA). The gels were scanned with a Scanner X finity Ultra (Quato Graphic, Braunschweig, Germany).

2.6 Protein quantitation

For protein quantitation, the 2-DE gel image analysis was performed with the software DELTA 2D version 3.4.1 beta 24 (Decodon GmbH, Greifswald, Germany). For each sampling point, gel images generated from three biological replicates were used to create a virtual fusion gel. This fusion gel was used for spot detection and spot mask editing. The final spot mask was transferred to each gel of the project to ensure 100% spot matching. The spot volumes of all protein spots obtained were normalized by using total normalization. The protein synthesis ratios of the bovicin HC5-stressed cells and the corresponding protein spot of the control cells were calculated. Significant changes were determined using Kruskal-Wallis Test ($p \leq 0.1$).

2.7 Protein identification

2.7.1 In gel digest

The protein spots were excised from stained 2-D gels using Ettan spot picker (GE Healthcare, United Kingdom) with a picker head of 2 mm diameter and transferred into 96 well micro titer plates (Greiner bio one, Austria). The tryptic digest with subsequent spotting on a MALDI-target was carried out automatically with the Ettan Spot Handling Workstation (GE Healthcare, United Kingdom) using the following protocol: the gel pieces were washed twice with 100 µl of a solution of 50% CH_3OH and 50% 50 mM NH_4HCO_3 for 30 min and once with 100 µl 75% CH_3CN for 10 min. After drying at 37^{o}C for 17 min, 10 µl trypsin solution containing 4 µg ml^{-1} trypsin (Promega, Madison, WI, USA) was added and incubated at 37^{o}C for 120 min. For extraction, gel pieces were covered with 60 µl 0.1% TFA in 50% CH_3CN and incubated

for 30 min at 40°C. The peptide containing supernatant was transferred into a new micro titer plate and the extraction was repeated with 40 µl of the same solution. The supernatants were dried at 40°C for 220 min completely. The dry residue was resuspended in 0.9 µl of α-cyano-4-hydroxycinnamic acid matrix (3.3 mg ml^{-1} in 50%/49.5%/0.5% [v/v/v] CH_3CN/H_2O/TFA) and 0.7 µl of this solution was deposited on the MALDI target plate. The samples were allowed to dry on the target 10 to 15 min before measurement in MALDI-TOF.

2.7.2 Mass spectrometry (MS)

The MALDI-TOF measurement was carried out on the AB SCIEX TOF/TOF™ 5800 Analyzer (AB Sciex / MDS Analytical Technologies, Germany). This instrument is designed for high throughput measurement, being automatically able to measure the samples, calibrate the spectra and analyze the data using the TOF/TOF™ Series Explorer™ Software V4.1.0. The spectra were recorded in a mass range from 900 to 3400 Da with a focus mass of 1700 Da. For one main spectrum 25 sub-spectra with 100 shots per sub-spectrum were accumulated using a random search pattern. If the autolytical fragment of trypsin with the mono-isotopic $(M+H)^+$ m/z at 2211.104 reached a signal to noise ratio (S/N) of at least 40, an internal calibration was automatically performed as one-point-calibration using this peak. The standard mass deviation was less than 0.15 Da. If the automatic mode failed (in less than 1%), the calibration was carried out manually. The five most intense peaks from the TOF-spectra were selected for MS/MS analysis. For one main spectrum, 20 sub-spectra with 125 shots per sub-spectrum were accumulated using a random search pattern. The internal calibration was automatically performed as one-point-calibration with the mono-isotopic arginine $(M+H)^+$ m/z at 175,119 or lysine $(M+H)^+$ m/z at 147,107 reached a signal to noise ratio (S/N) of at least 5.

2.7.3 MS data generation and database searching

The peak lists were created by using GPS Explorer™ Software Version 3.6 (build 332) with the following settings for TOF-MS: mass range, 900–3700 Da; peak density, 20 peaks per 200 Da; minimum S/N ratio of 15 and maximal 65 peaks per spot. The TOF-TOF-MS settings were a mass range from 60 to Precursor - 20 Da; a peak density of 50 peaks per 200 Da and maximal 65 peaks per precursor. The peak list was created for an S/N ratio of 10.

All peak lists were analysed using Mascot search engine version 2.1.04 (Matrix Science Ltd, London, UK) with a specific *S. aureus* COL sequence database.

Protein identification was performed as described previously or deduced from the 2D protein reference map (master gel) of *S. aureus* COL published elsewhere (Becher et al., 2009).

3. RESULTS AND DISCUSSION

3.1 Growth behavior of stressed cells of *S. aureus* by bovicin HC5

To investigate the growth effect of subinhibitory concentration of bovicin HC5 on *S. aureus* COL, exponentially growing cultures (OD_{500} = 0.5) were treated with 1.0 and 2.0 µM of the bacteriocin. Growth was followed by measuring the OD (Fig. 1). Addition of 1.0 µM bovicin HC5 to synthetic medium led to growth reduction after 30 min of exposition being the growth reassumed afterwards. The presence of bovicin HC5 at 2.0 µM showed growth inhibition of about 50% and this subinhibitory concentration was selected for global proteome analysis.

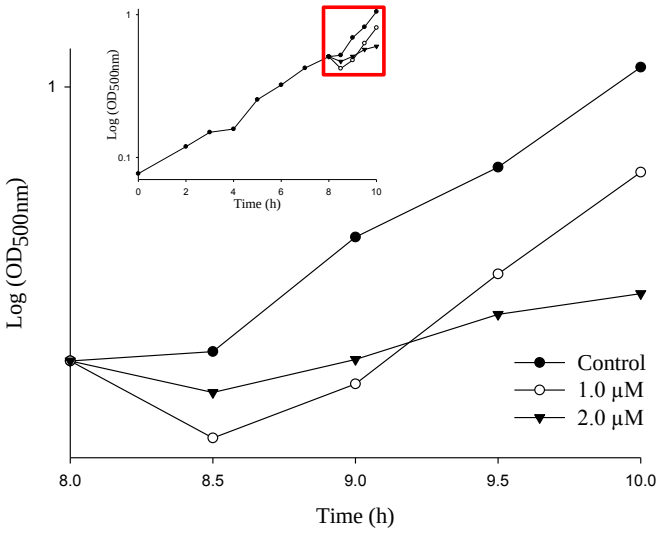

Figure 1. Growth inhibition of *S. aureus* induced by bovicin HC5. *S. aureus* COL was growth in synthetic medium at 37ºC and 150 rpm. At an OD_{500} of 0.5, the culture was split and treated with increasing amounts of bovicin HC5. Control represents untreated culture. The growth experiments were repeated three times. The results of one representative experiment are shown.

3.2 Effect of bovicin HC5 on global protein expression in *S. aureus*

Quantification of the synthesis of cytoplasmic proteins before and at different time points after exposure of *S. aureus* COL to 2.0 µM of bovicin HC5 was performed. All proteins whose abundance was significantly different when compared with untreated

control are shown in Tables 1 and 2. The analytical window of the 2D gel-based proteomic approach (pI range: 4 to 7; mass range: 10 to 140 kDa) covered 743 individual protein spots. Of these, the synthesis rate of 104 spots (62 proteins) was repressed whereas, at the same time, the synthesis rate of at least 87 spots (42 proteins) seemed to be induced. Out of these, identification was assigned to 106 protein spots by MS or master gel transfer. Since proteins may occur as multiple spots due to modifications changing their isoelectric points and/or molecular weights, the 106 spots were assigned to 104 individual proteins (Tables 1 and 2 and Figure 2). The remaining spots represent proteins that did not accumulate in detectable amounts in the cell and therefore are difficult to access for protein identification on 2D gels.

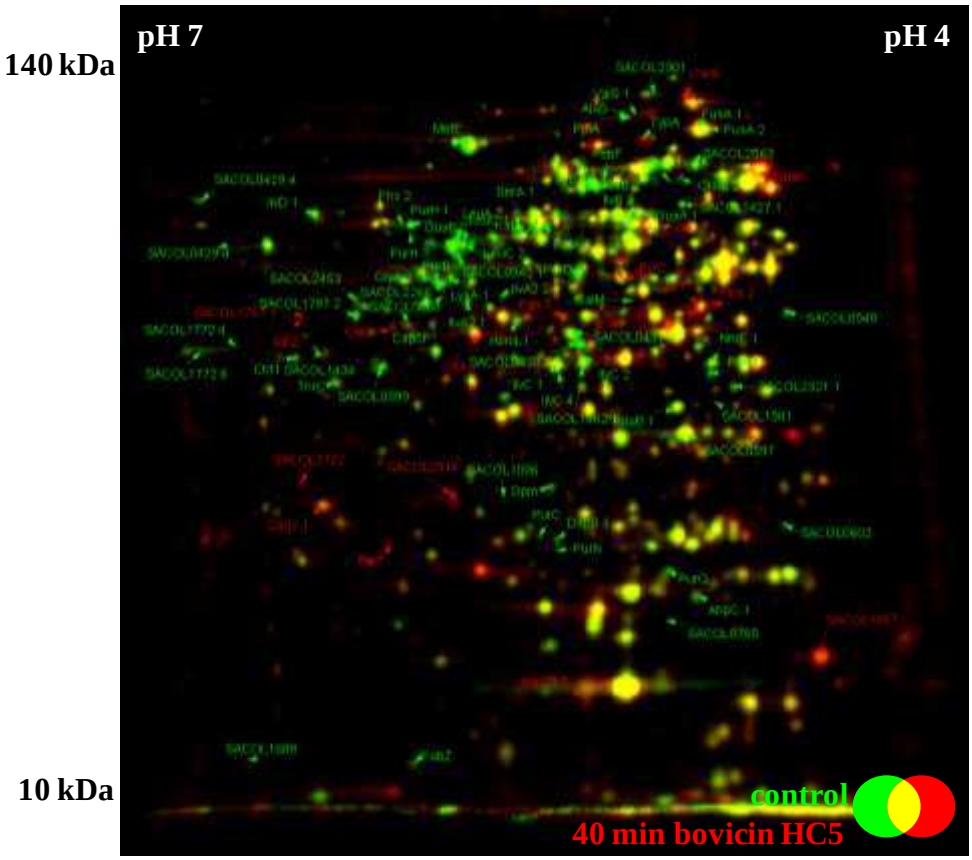

Figure 2. Dual-channel image of the synthesis of cytoplasmatic proteins following bovicin HC5 treatment. False-color image of 2D gels from L-[^{35}S]methionine-labeled protein extracts. Green, untreated control (exponentially growing cells); red, cells exposed to 2.0 µM bovicin HC5. As a representative example, a 2D gel from the 40 min stress time point is shown. The 2D gel images were overlaid and analyzed with Delta2D software. Proteins that were regulated at least at one time point during the course of the experiment are indicated. Proteins with significantly increased synthesis rates following bovicin HC5 treatment are labeled in red. The green-labeled spots represent proteins whose synthesis was significantly repressed. Proteins with unchanged synthesis rates in response to the treatment appear in yellow. Summaries of the regulated proteins are shown in Table 1 (repressed proteins) and Table 2 (induced proteins).

Table 1. Proteins whose synthesis rates were decreased after bovicin HC5 treatment

ORF ID *S. aureus* COL[a]	Symbol[a]	Spot ID	Co-localized with	Function[a]	Induction ratio after bovicin HC5 treatment[b] 10 min	20 min	40 min
Amino acid biosynthesis							
SACOL1787		SACOL1787 2		Chorismate mutase/phospho-2-dehydro-3-deoxyheptonate aldolase	-1.57	-0.73	-0.83
SACOL1431	DapB	DapB 1		Dihydrodipicolinate reductase	-1.26	-0.87	-0.85
SACOL1435	LysA	LysA 1		Diaminopimelate decarboxylase	-1.05	-0.48	-0.86
SACOL0428	MetE	MetE		5-Methyltetrahydropteroyltriglutamate--homocysteine Methyltransferase	-1.36	-2.49	-2.26
SACOL0429		SACOL0429 4		5-Methyltetrahydrofolate--homocysteine methyltransferase, putative	-1.80	-1.70	-2.78
SACOL0430		SACOL0430 1		Trans-sulfuration enzyme family protein	-2.11	-2.36	-2.48
SACOL0431		SACOL0431 3		Trans-sulfuration enzyme family protein	-1.26	-0.99	-1.02
SACOL1363	ThrC	ThrC		Threonine synthase	-1.40	-0.71	-0.84
SACOL2050	IlvA2	IlvA2 1		Threonine dehydratase	-3.24	-2.18	-1.75
		IlvA2 2			-0.68	-1.08	-1.92
SACOL2043	IlvB	IlvB 1	IlvB 2	Acetolactate synthase, large subunit, biosynthetic type	-1.41	-1.76	-1.50
		IlvB 2	IlvB 1		-1.41	-1.76	-1.50
		IlvB 3			-2.24	-2.40	-1.77
SACOL2045	IlvC	IlvC 1	IlvC 4	Ketol-acid reductoisomerase	-1.72	-1.25	-1.04
	IlvC	IlvC 2	SACOL1562 1		-1.02	-0.97	-0.84
	IlvC	IlvC 4	IlvC 1		-1.72	-1.25	-1.04
SACOL2042	IlvD	IlvD 1		Dihydroxy-acid dehydratase	-2.49	-1.71	-1.92
SACOL2046	LeuA	LeuA 2	GuaB 2	2-Isopropylmalate synthase	-2.30	-1.51	-1.89
SACOL2048	LeuC	LeuC 1	LeuC 2	3-Isopropylmalate dehydratase, large subunit	-2.74	-1.89	-2.11
		LeuC 2	LeuC 1		-2.74	-1.89	-2.11
SACOL1773	SerA	SerA 1		D-3-Phosphoglycerate dehydrogenase	-1.80	-1.31	-1.24
		SerA 2	KatA 2, KatA 5		-0.49	-0.49	-1.04
SACOL2105	GlyA	GlyA 3	PurB	Serine hydroxymethyltransferase	-1.33	-0.72	-1.14

Table 1. *(continued)*

ORF ID *S. aureus* COL[a]	Symbol[a]	Spot ID	Co-localized with	Function[a]	Induction ratio after bovicin HC5 treatment[b] 10 min	20 min	40 min
Biosynthesis of cofactors, prosthetic groups, and carriers							
SACOL1714	HemL1	HemL1		Glutamate-1-semialdehyde-2,1-aminomutase	-1.16	-0.47	-0.52
SACOL2266		SACOL2266		Molybdopterin biosynthesis MoeA protein, putative	-0.48	-0.45	-1.09
Cell envelope							
SACOL1434		SACOL1434		Alanine racemase family protein	-1.90	-1.02	-1.22
SACOL0151	Cap5P	Cap5P		UDP-N-acetylglucosamine 2-epimerase Cap5P	-0.41	-0.88	-1.43
Detoxification							
SACOL0452	AhpC	AhpC 1		Alkyl hydroperoxide reductase, C subunit	-0.64	-0.79	-1.07
SACOL1368	KatA	KatA 2	KatA 5, SerA	Catalase	-0.49	-0.49	-1.04
		KatA 5	KatA 2, SerA		-0.49	-0.49	-1.04
Central intermediary metabolism							
SACOL2145	GlmS	GlmS 1		Glucosamine--fructose-6-phosphate aminotransferase (isomerizing)	-0.87	-1.37	-0.61
		GlmS 2			-0.53	-1.44	-0.55
SACOL1782	Fhs	Fhs 2	PurH 1, PurH 3	Formate--tetrahydrofolate ligase	-0.95	-1.12	-2.10
DNA metabolism							
SACOL1737	PolA	PolA	AlaS, ValS	DNA polymerase I	-0.06	-1.07	0.33
Energy metabolism							
SACOL1561		SACOL1561		2-Oxoisovalerate dehydrogenase, E1 component, beta subunit	-0.76	-1.00	-0.39
SACOL1562		SACOL1562 1	IlvC 2	2-Oxoisovalerate dehydrogenase, E1 component, alpha subunit	-1.02	-0.97	-0.84
Anaerobic respiration							
SACOL2301		SACOL2301		Formate dehydrogenase, alpha subunit, putative	-0.42	-1.46	-0.79

Table 1. *(continued)*

ORF ID *S. aureus* COL[a]	Symbol[a]	Spot ID	Co-localized with	Function[a]	Induction ratio after bovicin HC5 treatment[b] 10 min	20 min	40 min
Glycolysis/gluconeogenesis							
SACOL2622	FdaB	FdaB 1		Fructose-bisphosphate aldolase, class I	-0.40	-0.72	-1.13
SACOL2415	Gpm	Gpm		Phosphoglycerate mutase	0.09	-0.33	-1.30
SACOL2332	GalM	GalM		Aldose 1-epimerase	-0.57	-0.92	-1.13
Fatty acid and phospholipid metabolism							
SACOL2091	FabZ	FabZ		Beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ	-0.60	-0.34	-1.04
Hypothetical							
SACOL0597		SACOL0597		Conserved hypothetical protein	-0.39	-0.84	-1.22
SACOL0785		SACOL0785		Conserved hypothetical protein	-2.05	-1.52	-1.09
SACOL1688		SACOL1688		Conserved hypothetical protein TIGR00256	-1.31	0.16	-0.23
Mobile and extrachromosomal element functions							
SACOL1898	Cbf1	Cbf1		Cmp-binding-factor 1	-1.56	-0.54	-0.84
Protein degradation							
SACOL1005	PepF	PepF		Oligoendopeptidase F	-0.91	-1.07	-0.28
SACOL0945		SACOL0945 1		Cytosol aminopeptidase	-1.14	-0.60	-1.20
SACOL2563		SACOL2563		ATP-dependent Clp protease, putative	-0.51	-2.08	-2.19
Protein synthesis							
SACOL0593	FusA	FusA 1		Translation elongation factor G	-0.15	-1.16	0.03
		FusA 2			-0.65	-1.60	-0.52
SACOL1673	AlaS	AlaS	PolA, ValS	Alanyl-tRNA synthetase	-0.06	-1.07	0.33
SACOL1710	ValS	ValS	AlaS, PolA	Valyl-tRNA synthetase	-0.06	-1.07	0.33
SACOL0793	NrdF	NrdF 1		Ribonucleoside-diphosphate reductase 2, beta subunit	-1.99	-1.79	-1.36

Table 1. *(continued)*

ORF ID *S. aureus* COL[a]	Symbol[a]	Spot ID	Co-localized with	Function[a]	Induction ratio after bovicin HC5 treatment[b] 10 min	20 min	40 min
Purine ribonucleotide biosynthesis							
SACOL0461	GuaA	GuaA 1		GMP synthase	-1.50	-1.29	-1.22
SACOL0460	GuaB	GuaB 2	LeuA 2	Inosine-5-monophosphate dehydrogenase	-2.30	-1.51	-1.89
SACOL1969	PurB	PurB	GlyA 3	Adenylosuccinate lyase	-1.33	-0.72	-1.14
SACOL1075	PurC	PurC		Phosphoribosylaminoimidazole-succinocarboxamide synthase	-0.44	-0.86	-1.31
SACOL1083	PurD	PurD		Phosphoribosylamine--glycine ligase	-0.88	-0.92	-1.43
SACOL1082	PurH	PurH 1	Fhs 2, PurH 3	Phosphoribosylaminoimidazolecarboxamide	-0.95	-1.12	-2.10
		PurH 3	Fhs 2, PurH 1	formyltransferase/IMP cyclohydrolase	-0.95	-1.12	-2.10
SACOL1080	PurM	PurM		Phosphoribosylformylglycinamidine cyclo-ligase	-1.16	-2.09	-2.98
SACOL1081	PurN	PurN		Phosphoribosylglycinamide formyltransferase	-1.36	-1.25	-2.05
SACOL1077	PurQ	PurQ		Phosphoribosylformylglycinamidine synthase I	-0.65	-0.70	-1.38
Transport							
SACOL2453		SACOL2453		Amino acid ABC transporter, ATP-binding protein	-1.42	-0.46	-0.65
SACOL1096		SACOL1096		TrkA potassium uptake family protein	-1.00	-0.64	-0.68
SACOL1427		SACOL1427 1		ABC transporter, ATP-binding protein	-1.69	-1.99	-1.31
Unknown function							
SACOL0399		SACOL0399		Oxidoreductase, putative	-0.44	-0.57	-1.32
SACOL0549		SACOL0549		Tetrapyrrole methylase family protein	-0.44	-1.25	-1.66
SACOL0602		SACOL0602		Hydrolase, haloacid dehalogenase-like family	-1.14	-1.30	-0.90
SACOL1772		SACOL1772 4		Aminotransferase, class V	-1.92	-0.92	-1.43
		SACOL1772 6			-2.56	-1.62	-2.55
SACOL2321		SACOL2321 1		Oxidoreductase, short chain dehydrogenase/reductase family	-0.41	-1.15	-1.46
SACOL0658		SACOL0658		HD domain protein	-1.06	-0.39	-1.15
SACOL1118	TypA	TypA		GTP-binding protein TypA	-0.63	-1.28	0.60
	NrdE	NrdE 2			-0.99	-1.33	-0.80

[a] The IDs, symbols and functions are based on the Institute for Genomic Research (TIGR) annotation (http://www.tigr.org).

[b] At the indicated time points following bovicin HC5 administration, radioactively labeled methionine was added for 5 min to visualize newly synthesized proteins. The induction ratios were calculated by dividing the normalized intensity of the protein spot in the 2D gel image at the indicated time point after bovicin HC5 stress (10, 20, or 40 min of stress) by the corresponding spot intensity in the image of the unstressed control at time point zero. Values are shown as $\log_2$ of ratios.

Table 2. Proteins whose synthesis rates were enhanced after bovicin HC5 treatment

ORF ID					Induction ratio after bovicin HC5 treatment[b]		
S. aureus COL[a]	Symbol[a]	Spot ID	Co-localized with	Function[a]	10 min	20 min	40 min
Amino acid biosynthesis							
SACOL1787		SACOL1787 1		Chorismate mutase/phospho-2-dehydro-3-deoxyheptonate aldolase	0.53	2.84	2.25
SACOL0431		SACOL0431 4		Trans-sulfuration enzyme family protein	0.18	0.38	1.34
Biosynthesis of cofactors, prosthetic groups, and carriers							
SACOL1052	MenD	MenD		2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid synthase/2-oxoglutarate decarboxylase	0.07	1.48	0.23
SACOL1820	RibD	RibD	SACOL1787 1	Riboflavin biosynthesis protein RibD	0.53	2.84	2.25
Toxin production and resistance							
SACOL0935	DltA	DltA 1		D-Alanine-activating enzyme/D-Alanine-D-alanyl carrier protein ligase	1.11	0.90	0.80
SACOL1942	VraR	VraR	SACOL2667	DNA-binding response regulator VraR	2.70	3.03	2.97
Central intermediary metabolism							
SACOL1912		SACOL1912 2		Glucosamine-6-phosphate isomerase, putative	0.56	1.37	1.39
SACOL1782	Fhs	Fhs 1		Formate--tetrahydrofolate ligase	0.95	1.54	1.03
Electron transport							
SACOL2534	Frp	Frp		NAD(P)H-flavin oxidoreductase	0.55	1.06	0.50

Table 2. *(continued)*

ORF ID *S. aureus* COL[a]	Symbol[a]	Spot ID	Co-localized with	Function[a]	Induction ratio after bovicin HC5 treatment[b] 10 min	20 min	40 min
Fermentation							
SACOL0241		SACOL0241		Alcohol dehydrogenase, zinc-containing	0.46	0.73	1.27
Glycolysis/gluconeogenesis							
SACOL0839	Pgk	Pgk 5		Phosphoglycerate kinase	0.63	1.13	1.32
TCA cycle metabolism							
SACOL1385	AcnA	AcnA 1		Aconitate hydratase	1.76	0.48	0.97
		AcnA 2			1.41	0.23	1.14
SACOL1742	GltA	GltA		Citrate synthase	1.66	2.63	2.33
SACOL1741	Icd	Icd 1		Isocitrate dehydrogenase, NADP-dependent	1.50	1.89	1.88
		Icd 2			1.12	1.82	2.26
SACOL1449	SucA	SucA 2		2-Oxoglutarate dehydrogenase, E1 component	1.19	0.57	1.27
Fatty acid and phospholipid metabolism							
SACOL0988		FabF	PyrC	3-Oxoacyl-(acyl-carrier-protein) synthase II	1.51	1.31	0.59
Hypothetical proteins							
SACOL1630		SACOL1630		Conserved hypothetical protein	1.24	1.00	1.02
SACOL2518		SACOL2518		Conserved hypothetical protein	1.72	2.64	2.67
Protein degradation							
SACOL0979	ClpB	ClpB 1		ATP-dependent Clp protease, ATP-binding subunit ClpB	0.22	-0.79	1.17
		ClpB 2			0.17	-0.32	2.01
SACOL0833	ClpP	ClpP 1	NusG	ATP-dependent Clp protease, proteolytic subunit ClpP	0.59	1.08	1.21
SACOL1419		SACOL1419		Oligoendopeptidase F, putative	0.09	-0.16	1.01

Table 2. *(continued)*

ORF ID *S. aureus* COL[a]	Symbol[a]	Spot ID	Co-localized with	Function[a]	Induction ratio after bovicin HC5 treatment[b] 10 min	20 min	40 min
Protein folding and stabilization							
SACOL1637	DnaK	DnaK		dnaK protein	0.96	1.19	2.82
SACOL2016	GroEL	GroEL		Chaperonin, 60 kDa	0.31	0.20	1.89
SACOL1638	GrpE	GrpE		Heat shock protein GrpE	0.89	1.10	2.51
Protein synthesis							
SACOL1685	Asp23	Asp23 2		Alkaline shock protein	0.80	1.03	1.32
		Asp23 5			0.83	1.30	1.10
SACOL1148	PheS	PheS		Phenylalanyl-tRNA synthetase, alpha subunit	0.59	1.36	1.18
SACOL1710	ValS	ValS 2		Valyl-tRNA synthetase	0.41	-0.10	2.43
Pyrimidine ribonucleotide biosynthesis							
SACOL1277	PyrH	PyrH		Uridylate kinase	0.50	1.16	0.82
SACOL1214	CarA	CarA		Carbamoyl-phosphate synthase, small subunit	1.57	1.84	1.10
SACOL1215	CarB	CarB		Carbamoyl-phosphate synthase, large subunit	2.99	2.10	2.10
SACOL1212	PyrB	PyrB		Aspartate carbamoyltransferase	2.73	2.76	1.72
SACOL1213	PyrC	PyrC	FabF	Dihydroorotase	1.51	1.31	0.59
SACOL1216	PyrF	PyrF		Orotidine 5-phosphate decarboxylase	2.32	2.65	1.42
Regulatory functions							
SACOL1786	CcpA	CcpA		Catabolite control protein A	0.04	0.98	1.18
SACOL1272	CodY	CodY 1		Transcriptional regulator CodY	0.36	1.38	1.13
		CodY 2			0.66	1.67	1.81
Transcription factors							
SACOL0582	NusG	NusG	ClpP 1	Transcription antitermination protein NusG	0.59	1.08	1.21

Table 2. *(continued)*

ORF ID *S. aureus* COL[a]	Symbol[a]	Spot ID	Co-localized with	Function[a]	Induction ratio after bovicin HC5 treatment[b] 10 min	20 min	40 min
Transport							
SACOL1457		SACOL1457		PTS system, IIA component	1.65	2.03	2.44
SACOL1952		SACOL1952 1		Ferritins family protein	1.58	1.74	3.14
Unknown function							
SACOL0976		SACOL0976		Hydrolase, haloacid dehalogenase-like family	0.37	0.85	1.01
SACOL2667		SACOL2667	VraR	Isochorismatase family protein	2.70	3.03	2.97
SACOL2722		SACOL2722		N-acetyltransferase family protein	0.43	1.29	0.69
SACOL2499		SACOL2499		Helicase, putative	1.17	1.31	0.83

[a] The IDs, symbols and functions are based on the Institute for Genomic Research (TIGR) annotation (http://www.tigr.org).

[b] At the indicated time points following bovicin HC5 administration, radioactively labeled methionine was added for 5 min to visualize newly synthesized proteins. The induction ratios were calculated by dividing the normalized intensity of the protein spot in the 2D gel image at the indicated time point after bovicin HC5 stress (10, 20, or 40 min of stress) by the corresponding spot intensity in the image of the unstressed control at time point zero. Values are shown as $\log_2$ of ratios.

Among the proteins with repressed synthesis pattern following by bovicin HC5 treatment are a prominent group of proteins involved in amino acid biosynthesis, mainly pyruvate family (Figure 3), but also some enzymes of aspartate and serine families (Table 1). The stress caused by bovicin HC5 was more pronounced in the first 30 min after exposition where the growth was totally inhibited being reassumed in a reduced ratio afterwards (Figure 1), indicating adaptation phase. Reduction of proteins synthesis required for amino acid biosynthesis occurred strongly after the first 10 min of stress. Although co-localized in the gel with other proteins, synthesis of an important enzyme involved in cell multiplication, DNA polymerase I (PolA), seems to be reduced. These finds support the idea that bacterial cell reduces its growth and multiplication by decreasing protein synthesis immediately under stress condition. All these enzymes of pyruvate family biosynthesis are product of *ilv-leu* operon (*ilvDBNC leuABCD ilvA*) and regulated by the transcriptional factor CodY. CodY, first described in *Bacillus subtilis*, is a highly conserved regulatory protein of stationary phase adaptation in low G+C gram-positive bacteria, including *S. aureus* (Pohl et al., 2009; Stenz et al., 2011). It is known that *B. subtilis* CodY regulates more than 100 genes, which products are generally involved in the bacterial adaptation to poor growth conditions, such as extracellular degradative enzymes, transport systems, catabolic pathways, genetic competence, antibiotic synthesis, flagellin and early sporulation functions (Sonenshein, 2005). The synthesis of this repressor was induced in *S. aureus* COL during bovicin HC5 stress (Figure 4) and it seems to be active based on its function to repress synthesis of branched-chain amino acids metabolism enzymes. It is already known that bovicin HC5 acts binding to lipid II and forming pores in the plasmatic membrane that can disrupt the proton-motive force and cause efflux of intracellular potassium, leading to the collapse of ions gradients across the membrane (Mantovani and Russell, 2008; Paiva et al., 2011). Perturbation caused by damages on the cell envelope interferes with cell homeostasis leading to activation of a response with direct impact on adaptation and resistance. Pores formed by nisin, another lantibiotic bacteriocin, allow rapid outflow of small cytoplasmatic compounds, such as amino acids and ATP (Martin and Breukink, 2007). Considering that CodY senses nutrient availability by direct interaction with metabolite effectors (isoleucine and GTP) which independently and additively increase the affinity of CodY for its DNA target sites (Majerczyk et al., 2010), a speculative hypothesis would be that the membrane pores formed by bovicin HC5 allow amino acids entrance, depending on their concentration, that would act as CodY effectors

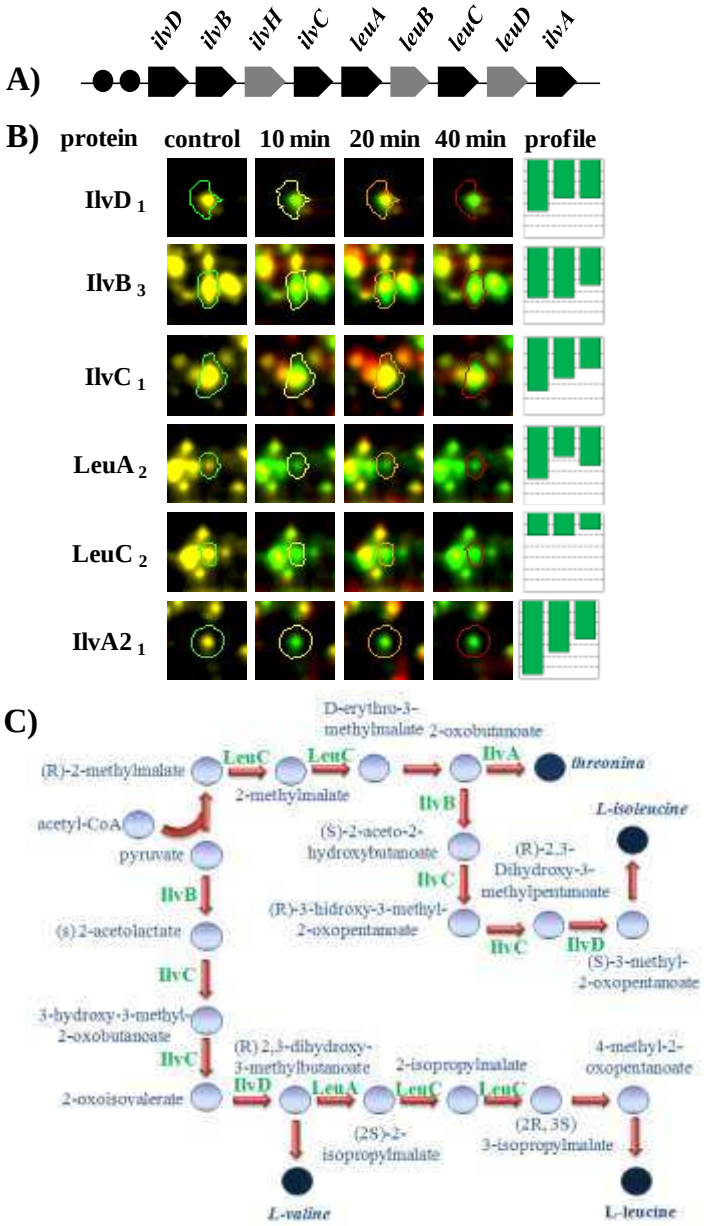

Figure 3. Effect of bovicin HC5 on pyruvate family amino acid biosynthesis pathway. (A) Schematic representation of the *ilv-leu* operon based on the sequence of *S. aureus* COL and the transcriptional organization are shown. Genes are shown by arrows. Black arrows are genes which translated proteins were identified by MS or transferred by master gel. Transcriptional factor binding sites are shown by black cycles. Note: IlvH (molecular weight of 9.62 kDa) is out of the analytical window. (B) Synthesis pattern of the proteins involved on BCAAs, Ala, and Thr pathway, under non-stressed condition at exponential growth (control) compared with the protein synthesis patterns at different time points after addition of bovicin HC5 (10, 20, and 40 min). Green indicates repressed synthesis. The diagrams on the right illustrate fold changes in protein synthesis rate as $\log_2$ values of stress/control ratio protein spot intensities at different time points (10 min/control, 20 min/control, 40 min/control). If a protein was present as a multiple spots on 2D gels, the respective spot identifier (ID) is shown as a subscript. (C) Schematic representation of pyruvate family amino acid biosynthesis pathway. Enzymes which synthesis decreased in the presence of bovicin HC5 are shown in green.

turning this repressor more active. On the other hand, the fact that the highest synthesis of CodY occurs by 20 min after bovicin HC5-stress does not completely explain the phenotype found that synthesis of proteins involved in amino acids biosynthesis was strongly repressed just 10 min after bovicin HC5 treatment. Thus, another regulatory network may be active. Since *ilv-leu* operon is regulated also in response to leucine availability by the T-box transcription antitermination system (Grandoni et al., 1993), tests must be done to study the effect of the bacteriocin on the GTP level and amino acid concentration inside and outside of the cell.

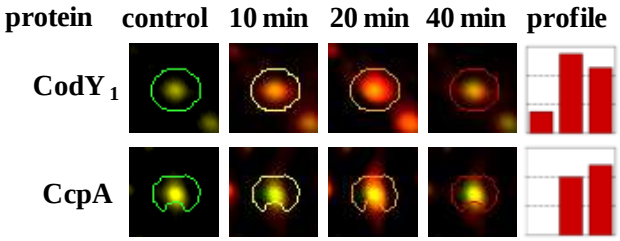

Figure 4. Effect of bovicin HC5 on synthesis of CodY and CcpA by *S. aureus* COL. Synthesis pattern of CodY and CcpA under non-stressed condition at exponential growth (control) compared with the protein synthesis patterns at different time points after addition of bovicin HC5 (10, 20, and 40 min). Red indicates induced synthesis. The diagrams on the right illustrate fold changes in protein synthesis rate as $\log_2$ values of stress/control ratio protein spot intensities at different time points (10 min/control, 20 min/control, 40 min/control). As CodY was present as a multiple spots on 2D gels, the respective spot ID is shown as a subscript.

Following bovicin HC5 treatment, a variation in the synthesis profile of some proteins of energy metabolism was observed. Synthesis of the glyconeogenesis enzymes frutose-biphosphate aldolase (FdaB), phosphoglycerate mutase (Gpm), and aldose 1-epimerase (GalM) decreased. On the other hand, synthesis of phosphoglycerate kinase (SACOL0839), another important glycolytic enzyme, was induced during bovicin HC5 stress. While enzymes involved with amino acids and amines metabolism (SACOL1561 and SACOL 1562) and anaerobic respiration (SACOL2301) were under-expressed, the abundance of enzymes related to electron transport (SACOL2534), fermentation (SACOL0241), and tricarboxylic acid (TCA) cycle (AcnA, GltA, Icd, and SucA) increased (Table 1 and Table 2, Figure 5).

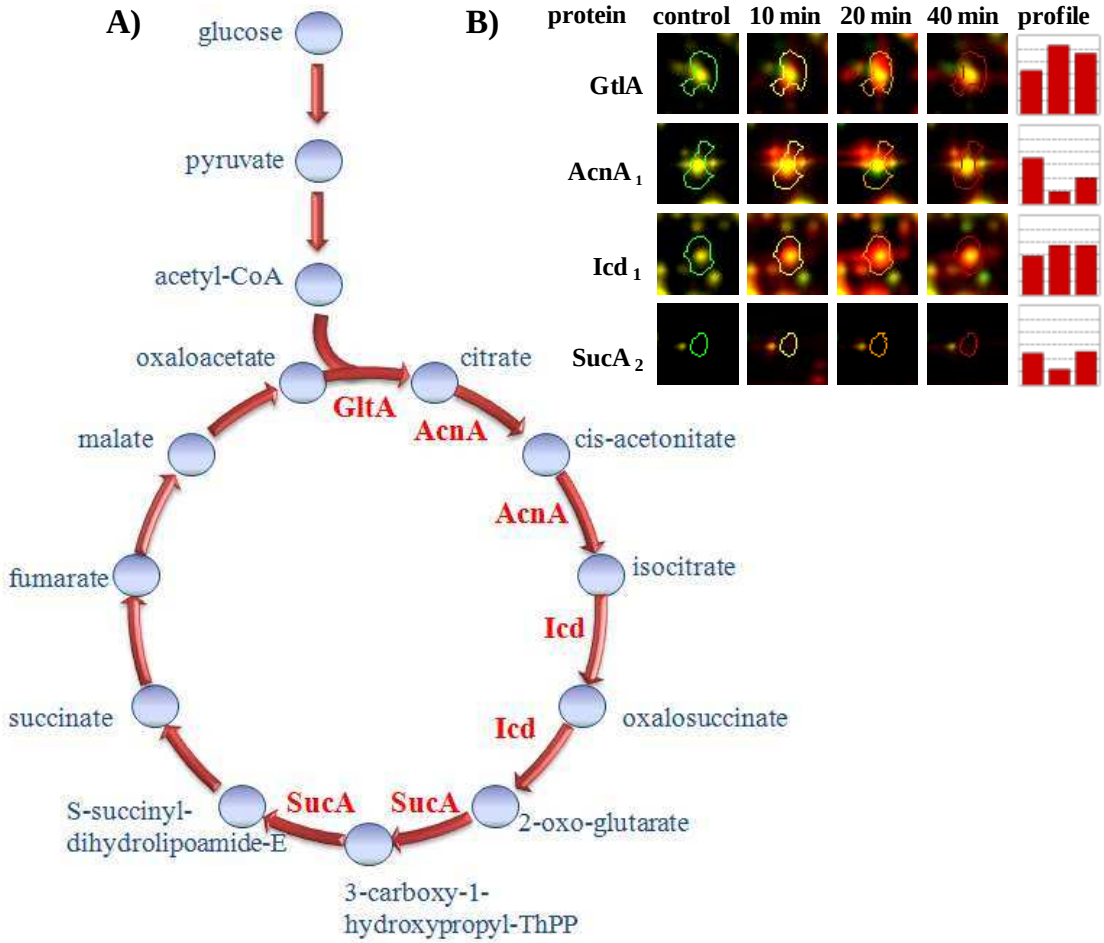

Figure 5. Effect of bovicin HC5 on TCA cycle proteins of *S. aureus* COL. (A) Schematic representation of TCA cycle. Enzymes which synthesis enhanced in the presence of bovicin HC5 are shown in red. (B) Synthesis pattern of the proteins involved on TCA cycle, under non-stressed condition at exponential growth (control) compared with the protein synthesis patterns at different time points after addition of bovicin HC5 (10, 20, and 40 min). Red indicates induced synthesis. The diagrams on the right illustrate fold changes in protein synthesis rate as $\log_2$ values of stress/control ratio protein spot intensities at different time points (10 min/control, 20 min/control, 40 min/control). If a protein was present as a multiple spots on 2D gels, the respective spot ID is shown as a subscript.

Although some proteins involved in energy metabolism showed decreased synthesis pattern, many proteins involved in catabolism showed induced synthesis level. These enzymes involved in TCA cycle are regulated by CcpA (catabolite control protein A), which synthesis enhanced in the presence of bovicin HC5 (Figure 4). CcpA is known to function as a major regulator of gene expression in different gram-positive organisms. It functions as a catabolite repressor or activator, allowing the bacteria to utilize the preferred carbon source over secondary carbon sources (Moreno et al., 2001;

Seidl et al., 2009). Our results is an indication that following stress caused by bovicin HC5, *S. aureus* enhances catabolic pathways in attempt to generate and restore energy for synthesis of crucial proteins involved and cell adaptation and survival.

In a total, five heat-inducible chaperones and proteases (GprE, DnaK, GroEL, ClpP, and ClpB) were induced in *S. aureus* COL following bovicin HC5 treatment showing the higher synthesis ratio after 40 min of exposure to the bacteriocin (Figure 6). In fact, the survival of *S. aureus* under unfavorable stress conditions that are critical for its persistence in the environment and for pathogenicity is facilitated by a variety of different mechanisms (Singh et al., 2012). Heat shock proteins are molecular chaperones and proteases highly synthesized during sublethal heat and also under other stress conditions assisting in protein folding and renaturation, degradation of improperly folded proteins, and also preventing protein aggregation (Lund, 2009; Singh et al., 2007). All of these proteins are controlled by the repressor of the heat shock response CtsR being GprE, DnaK and GroEL co-regulated by HrcA (Chastanet et al., 2003). Although fitting in the 2D gels analytical window, both transcriptional factors did not show significant differences in the synthesis pattern in the presence of bovicin HC5. Singh et al. (2007) demonstrated that a non-functional DnaK system reduced tolerance to heat, oxidative and antibiotic stresses in *S. aureus*. A fourfold reduction in the oxacillin and meticillin minimal inhibitory concentration was observed for the *dnaK* mutant when compared with the wild-type *S. aureus* COL suggesting that DnaK plays a role in dealing with damaged proteins resultant of cell wall-active antibiotics (Singh et al., 2007). The chaperone GroEL also seems to be part of the cell wall-active antibiotic stress response in *S. aureus* RN 450 since the transcription of *groEL* was enhanced in response to oxacillin (Singh et al., 2001). As observed in bovicin HC5 treated cells, the synthesis of DnaK, GroEL, GrpE, ClpP and ClpB was induced by the oxidative stress caused by diamide (Wolf et al., 2008). The enhanced synthesis of these chaperones indicates that they play a role in the adaptation of *S. aureus* to the stress caused by the pore-forming bacteriocin bovicin HC5.

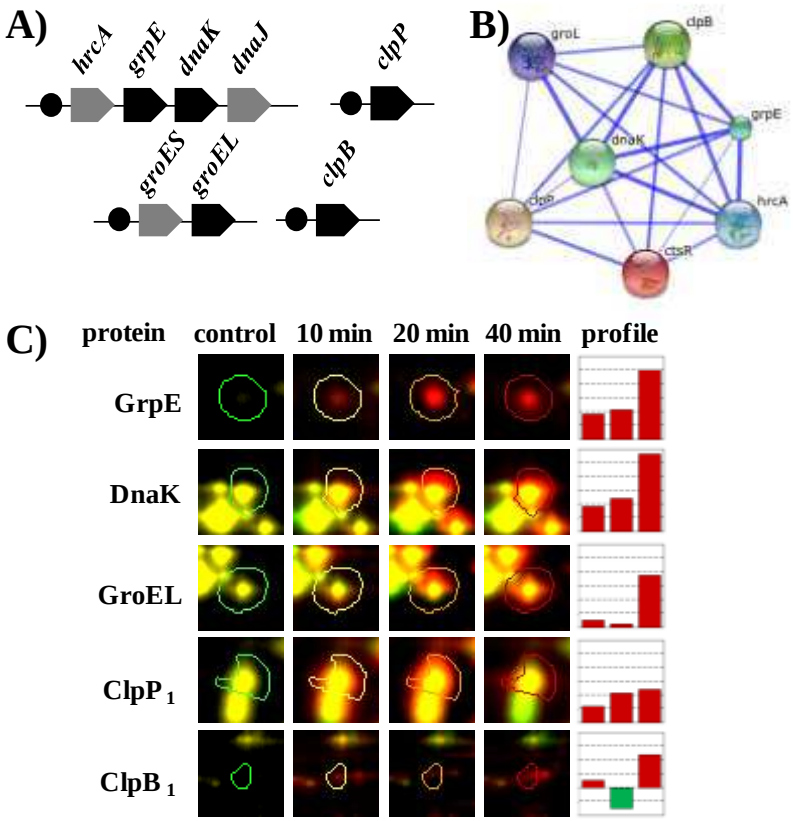

Figure 6. Synthesis pattern of significantly induced proteins involved in heat shock response (chaperones) of *S. aureus* COL following bovicin HC5 treatment. (A) Schematic representations of operons based on the sequence of *S. aureus* COL and the transcriptional organization. Genes are shown by arrows. Black arrows are genes which translated proteins were identified by MS or transferred by master gel. Transcriptional factor binding sites are shown by black cycles. (B) Confidence view of over-expressed chaperones and proteases during bovicin HC5 stress and their interaction with the repressor regulators CtsR and HrcA. Stronger associations are represented by thicker lines. Image was built using the online data base String 9.1 (http://www.string-db.org/). (C) Shown are false-color images of the protein synthesis rate of *S. aureus* COL grown in synthetic medium under non-stressed conditions (exponential growth) (control) compared to the protein synthesis patterns at different time points after the addition of bovicin HC5 (10, 20, and 40 min). Red indicates induced synthesis. The diagrams on the right illustrate fold changes in protein synthesis rate as $\log_2$ values of stress/control ratio protein spot intensities at different time points (10 min/control, 20 min/control, 40 min/control). If a protein was present as a multiple spots on 2D gels, the respective spot ID is shown as a subscript.

Enhanced synthesis of DltA (D-alanine-activating enzyme/D-alanine-D-alanyl carrier protein ligase) was observed as a resistance response to the presence of bovicin HC5. For membrane damage, which is considered the primary antimicrobial mechanism, antimicrobial peptides, such as bacteriocins, require cationic properties for

the initial interaction with the negatively charged membrane surface (Peschel and Sahl, 2006). In *S. aureus*, products of *dlt* operon, which include the proteins DltA, DltB, DltC and DltD, partially neutralize the negative net charge of the cell surface by modifying teichoic acid with D-alanine residues that bear positively charged amino groups (Peschel et al., 1999). These modifications hamper interaction between bacteriocins, positively charged peptides, and the surface of target cells.

The synthesis ratio of other proteins involved in cell detoxification, alkyl hydroperoxide reductase (AhpC) and KatA (catalase), also regulated by CodY, decreased in the presence of bovicin HC5. Instead of that, the oxidant agents H_2O_2, paraquat, and diamide induced AhpC synthesis while mupirocin enhanced both AhpC and KatA accumulation (Reiß et al., 2011; Wolf et al., 2008).

Surprisingly, most of the proteins translated from the ribonucleotide operons were differentially expressed. But while enzymes of purine biosynthesis were under-expressed following bovicin HC5 treatment, pyrimidine biosynthesis proteins were up-regulated (Figure 7). In accordance with our findings, a strong reduction of purine metabolism enzymes synthesis (*pur* operon) was observed in *S. aureus* stressed by mupirocin, a drug used for nasal staphylococcal decolonization (Reiß et al., 2011). In the same study, the authors found only one protein involved in pyrimidine biosynthesis (PyrF) under-expressed in the presence of mupirocin. In an opposite way, compared to *S. aureus* P100, a more sensitive strain to vancomycin (MIC of 2 mg ml^{-1}), VP32 strain (MIC of 32 mg ml^{-1}) showed strong increases in purine ribonucleotide biosynthesis enzymes (Pieper et al., 2006). This decreasing in the synthesis level of so many proteins involved in purine biosynthesis is an indication that bovicin HC5 can cause a stringent response such as mupirocin. Stringent response is characterized by a massive repression of genes whose products are required at a high level during rapid growth to adjust cells biosynthetic machinery to the new condition (Reiß et al., 2011). We have not an experimentally verified explanation for the relationship between purine ribonucleotide biosynthesis enzymes reduction combined with high expression of pyrimidine biosynthesis enzymes and the stress caused by bovicin HC5. It is still a matter of discussion why only genes whose products are involved in purine metabolism, but not in pyrimidine metabolism, were repressed.

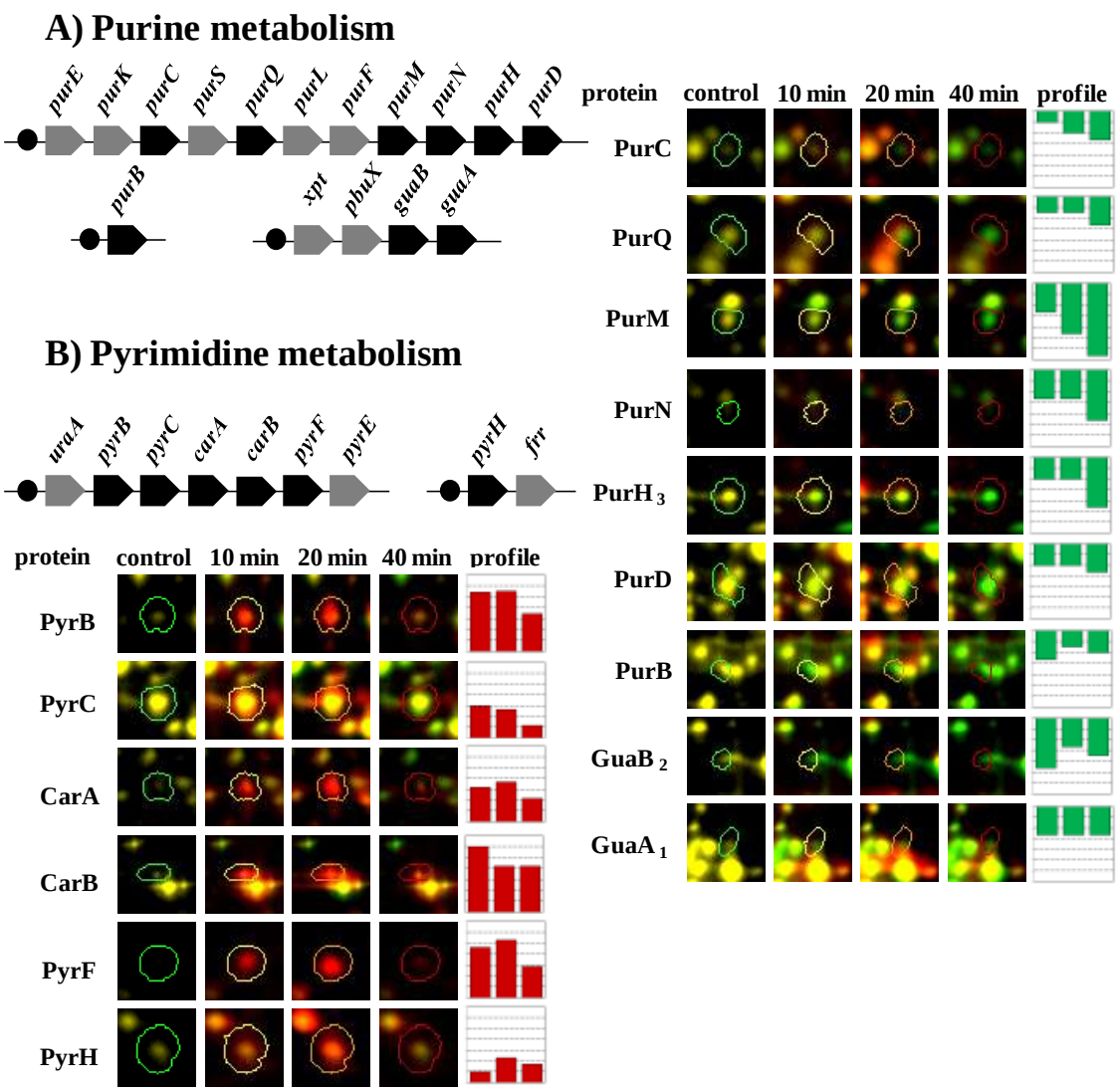

Figure 7. Timeline analysis of newly synthesized ribonucleotide biosynthesis proteins by *S. aureus* COL following bovicin HC5 treatment. Shown are false-color images of the protein synthesis rate of S. *aureus* COL grown in synthetic medium under non-stressed conditions (exponential growth) (control) compared to the protein synthesis patterns at different time points after the addition of bovicin HC5 (10, 20, and 40 min). Green indicates repressed synthesis, while red, induced synthesis. The diagrams on the right illustrate fold changes in protein synthesis rate as $\log_2$ values of stress/control ratio protein spot intensities at different time points (10 min/control, 20 min/control, 40 min/control). If a protein was present as a multiple spots on 2D gels, the respective spot ID is shown as a subscript. Also shown are schematic representations of purine and pyrimidine operons based on the sequence of *S. aureus* COL and the transcriptional organization. Genes are shown by arrows. Black arrows are genes which translated proteins were identified by MS or transferred by master gel. Transcriptional factor binding sites are shown by black cycles. (A) Protein synthesis of enzymes of purine ribonucleotide biosynthesis was repressed following bovicin HC5 addition. (B) Protein synthesis of enzymes of pyrimidine ribonucleotide biosynthesis was induced.

4. CONCLUSIONS

This work described the physiological response of *S. aureus* COL to the bacteriocin bovicin HC5. As evidenced by the global protein expression profile (Figure 8), the synthesis of many proteins involved in amino acids biosynthesis and DNA metabolism decreased following bovicin HC5 treatment while proteins involved in catabolism and chaperones were over-expressed. These results indicate that stress caused by bovicin HC5 reduces protein biosynthesis stopping cell growth and shifting toward a more energy-efficient catabolism, possibly triggered by a lowered energy state in the cells induced by membrane damages in attempt to restore the cell homeostasis.

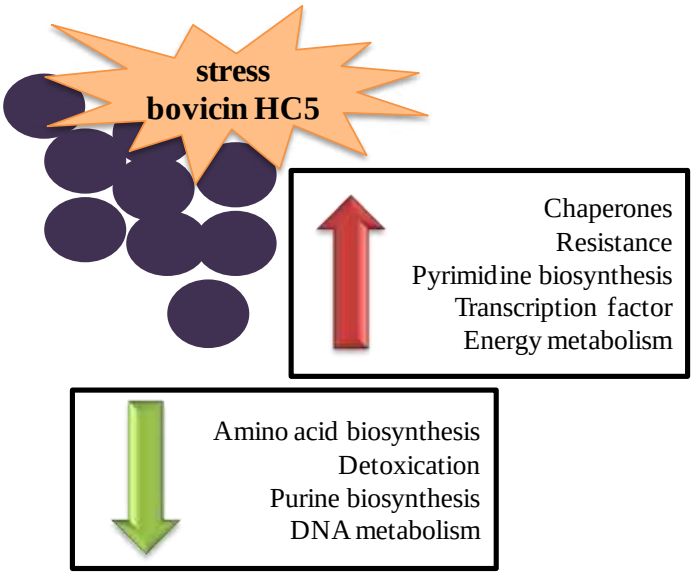

Figure 8. Schematic representation of the effect of bovicin HC5 on the protein expression in *S. aureus* COL. Green arrow indicates protein groups with decreased synthesis; Red, protein groups with increased synthesis.

In a metabolic overview, aspartate is highlighted as a key molecule for *S. aureus* COL response to the presence of subinhibitory concentration of bovicin HC5 (Figure 9). This amino acid is required for many metabolic pathways and cellular processes suggesting its involvement in the bacterial adaptation and resistance to the stress caused by bovicin HC5.

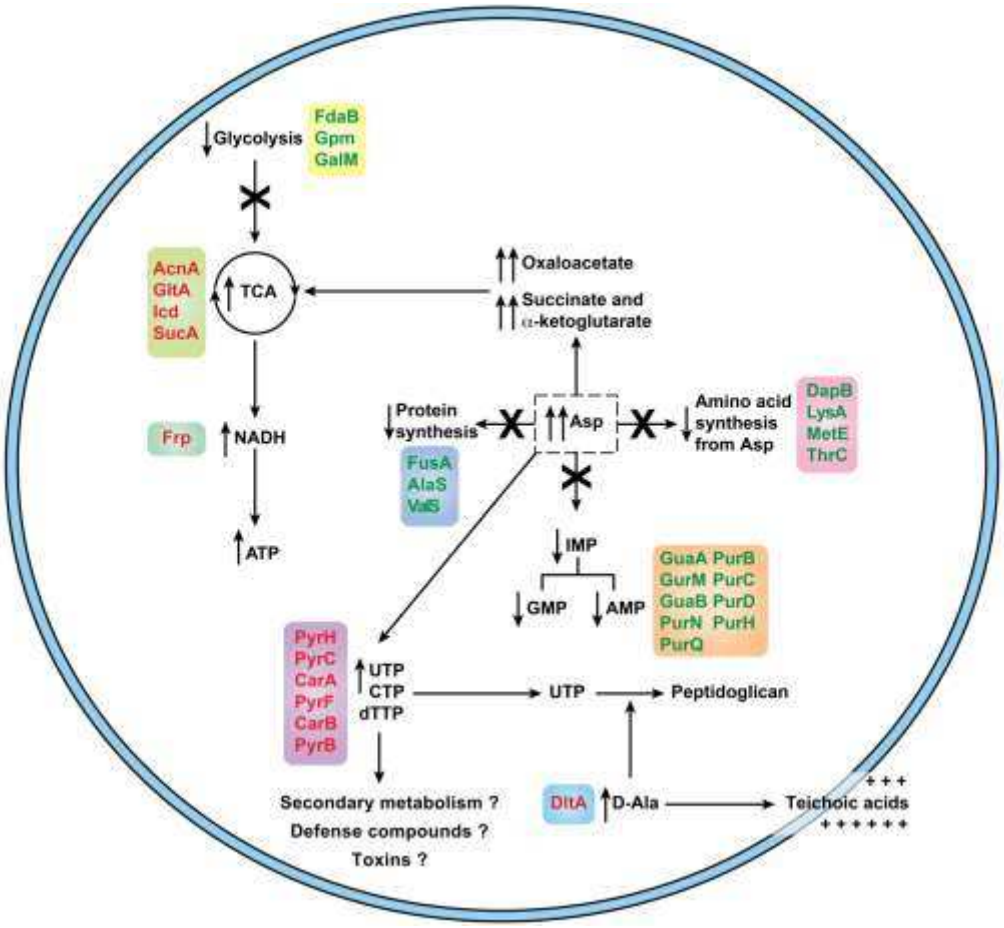

Figure 9. Interaction of metabolic pathways proposed for *S. aureus* COL in response to bovicin HC5. Synthesis of enzymes involved in biosynthesis of general proteins (dark blue box), purine (orange box) and amino acids (especially of pyruvate and aspartate families (pink box)) decreased in the presence of the bacteriocin meanwhile synthesis of enzymes involved in pyrimidine biosynthesis (purple box) enhanced. Aspartate seems to be a key molecule for stress response of *S. aureus* to subinhibitory concentration of bovicin HC5. The variation in the protein synthesis pattern suggests that, by anaplerotic reactions, aspartate can activate synthesis of TCA cycle intermediates such as oxaloacetate, α-ketoglutarate, and succinate which was evidenced by enhanced synthesis of enzymes involved in the Krebs cycle (light green box). Increased synthesis of flavin reductase protein (dark green box) is another indication that TCA cycle is induced, since this pathway contributes to the production of reduced coenzymes and APT, important for energy generation and cell homeostasis maintenance. This finding is corroborated by decrease in the synthesis of enzymes from glycolysis pathway (yellow box), suggesting that energy generation is not originate from glucose oxidation. Furthermore, participation of aspartate in the purine nucleoside synthesis may initiate defense mechanisms responses, for example the requirement of UTP in D-Ala synthesis (light blue box), important for peptidoglycan production and teichoic acids modifications that enhance cell resistance to the action of cationic peptides such as bovicin HC5. Proteins with repressed synthesis following bovicin HC5 treatment are shown in green. Proteins with enhanced synthesis following bovicin HC5 treatment are shown in red.

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GENERAL CONCLUSIONS

In this thesis, subinhibitory concentrations of the bacteriocins bovicin HC5 and nisin showed an interesting effect on adhesion of *S. aureus* to polystyrene surface. Even not killing cells, both bacteriocins were able to reduce cell adhesion. The free energy of adhesion was unfavorable since bovicin HC5 and nisin changed the hydrophobicity of cells and polystyrene surfaces. On the other hand, biofilm associated genes were up-regulated in the presence of bovicin HC5 and nisin. It is an indication that free energy of adhesion affects more *S. aureus* adhesion than synthesis of biofilm appendages such as IcaD and FnbA. These findings suggest that conditioning of food-contact surfaces with bacteriocins could be a powerful strategy to prevent biofilms in the food industry avoiding risks of food cross-contamination. Another innovative option to be evaluated could be include bacteriocin solutions as a sanitizing step during equipment cleaning.

Bovicin HC5 disturbed cell homeostasis of *S. aureus* COL leading to a stress response. Protein synthesis of *S. aureus* changed following bovicin HC5 treatment. While biosynthesis of amino acids was decreased, chaperones and enzymes involved in catabolic pathways were over-expressed. Synthesis of DltA can be an indication of resistance response. In the presence of bovicin HC5, enzymes of purine biosynthesis pathway were under-expressed whereas pyrimidine biosynthesis proteins were up-regulated. To our knowledge, this is the first report of a complete overview of protein synthesis in cells of *S. aureus* stressed by bacteriocins. These findings improve the understanding of stress response caused by bovicin HC5. Further, proteomic analysis of the effect of bovicin HC5 on membrane fraction proteins would be performed in order to complement our study.